Media Optimization for the production of lipopeptide from *Streptomyces iconiensis* VR1 Strain

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Abstract
This study reports a suitable culture medium for lipopeptide production using different nitrogen and carbon sources. Lipopeptide was produced from Streptomyces iconiensis VR1 Strain isolated from a soil sample. Sorbitol was the most suitable carbon source, compared to glucose, starch, fructose, maltose and sucrose. The beef extract was found to be a potential source of nitrogen for lipopeptide production. Therefore, a pH of 7, temperature of 30(°C) and time (72h) showed maximum lipopeptide production yield.

The lipopeptide extract obtained from streptomyces icoiniensis showed the highest antifungal activity against Mucor sp. with 70.8 ± 2.6% and Aspergillus niger 45.3 ± 1.5% of zone inhibition. Lipopeptide extract showed significant cell viability (94.68 ± 2.2%) at 75 g/mL against Human cervical HT-3 cancer cells.

Keywords: Media Optimization, Lipopeptide, Antifungal activity, MTT Assay.

Introduction

The glycolipids, lipopeptides, polysaccharides, proteins and lipoproteins are structurally diverse biosurfactants discovered so far[1-6]. A short linear (or) cyclic oligopeptide is joined to a lipid tail to form a lipopeptide (LP). The lipopeptide was produced from several bacterial and fungal strains such as *Streptomyces*, *Pseudomonas*, *Bacillus* and *Aspergillus*. Lipopeptides possess various biological activities like antibacterial, cytotoxic, antitumor, immunosuppressive and surfactant activities etc.[7–10] These biosurfactants (BS) contain surface-active compounds of microbial origin and result in various applications in business, medicine, (or) agriculture.

Lipopeptides (LPs) are the most important group present in the biosurfactants along with lipopolysaccharides, phospholipids and glycolipids. Several studies reported the potential uses of biosurfactants[11–16]. In addition to the pharmaceutical and petroleum industries, lipopeptides are utilized in the fields of agriculture, environmental remediation, cosmetics and food preparation. Lipopeptides produced from bacteria, filamentous fungi and yeasts are used as effective emulsifiers, low toxins, foaming agents, dispersers and surface alteration reagents[17,18]. Due to their distinctive properties such as emulsification, detergency, synthetic detergents, rusting inhibition and lubricant, biosurfactants are important compounds for many industries[19].

Furthermore, *Pseudomonas sp.* and *Bacillus sp.* are the primary bacteria used for the production of lipopeptides[20-22]. The maximal lipopeptide production is based on the reaction parameters of lipopeptide production which can be controlled inside a specific set of operating conditions like pH, salinity, medium composition, temperature etc.[23,24] The production of biosurfactants was studied based on the carbon and nitrogen sources, carbon-to-nitrogen ratios, mineral component concentrations, pH, inoculum size, agitation, temperature etc.[25,26]. Depending on their structural differences, various sets of lipopeptide surfactants are classified, based on the amino acid sequence[27,28]. Numerous bacteria have been identified for the production of a variety of biosurfactants including polymeric, particle surfactants, phospholipids, lipopeptides and glycolipids[29].

However, the features of the biosurfactants produced by various strains vary including surface tension decrease, productivity, emulsifying efficiency and durability[30]. The lipopeptides produce less bacterial resistance than conventional antibacterial agents and are eco-friendly[31,32]. In this study, the optimization of lipopeptide productivity was reported using *Streptomyces iconiensis* isolated from a soil sample. Using different carbon and nitrogen sources, beef extract and sorbitol show enhanced lipopeptide production.

Material and Methods

Screening and isolation of a bacterial strain from a soil sample: The soil sample was collected from the seashore place of Tuticorin, Tamil Nadu, India in a sterile bag under aseptic conditions. Using the serial dilution method, the soil samples were prepared by dilution of 10^{-1} to 10^{-10} times at pH 7.2 of nutrient broth. Take 30µl of every sample dilution and spread it over on the nutrient agar Petri plates. From LP-1 to LP-7, seven distinct colonies of bacterial isolates from the agar plate have been tested for antifungal activity. The isolated single bacterial species (LP-3) exhibited a significant zone of inhibition activity with high sensitivity towards the fungal strain such as *Aspergillus niger* and *Mucor* species selected for further experimental studies.

The selected LP-3 isolates were cultivated in 250ml Erlenmeyer flasks with Luria Bertani broth and maintained with an optimum pH of 7 and temperature of 30°C at 200rpm in a shaker for 72 hours. Produced lipopeptide was centrifuged from the fermenting LB broth (10,000 g for 10 min at 4°C). After collecting the cell-free broth, 6 N HCl was

used to maintain a pH of 2. Methanol was used to extract LP collected by centrifugation process (12,000 g at 4 °C for 20 min).

Evaluation of antifungal activity: The agar well-diffusion technique was employed to assess the antifungal activity of lipopeptide extract (50 µL) against *A. niger* and *Mucor sp*.[34] In the center of the PDA center test, a fungal culture was placed and a suitable quantity of methanol-extracted lipopeptide mixture was added under aseptic conditions into the well (diameter 6 mm).After three days of incubation at 30°C, the Petri plates were evaluated for the zones of resistance (%) against *A. niger* and *Mucor sp*.

Molecular Characterization - 16S rRNA sequencing for LP-3 bacterial isolate: Utilizing a commercially available kit, Endopore staining and Gram staining of the isolated bacteria species were carried out (Hi Media, Mumbai, India). 16S rRNA sequencing was used to analyze the chosen bacterial isolate LP-3. With the aid of forward and backward sequences, sequences of genes associated with bacterial strains are downloaded in the database *via* BLAST and used in the CLUSTAL-W tool of software MEGA version 6[35]. The neighbor-joining technique was employed to determine an evolutionary dendrogram. The dendrogram and evolutionary background of the taxa examined were shown using the 500 replicates of the Bootstrap consensus tree[36]. The DNA sequence was submitted to Genbank.

Media Optimization using different nitrogen, carbon sources, pH, production time and temperature: Media culture condition was optimized using different parameters like 1% (w/v) of different nitrogen sources like ammonium sulphate, ammonium nitrate, peptone, ammonium chloride, beef extract, sodium nitrate, or yeast extract and different 1% (w/v) carbon sources like glucose, fructose, starch, sorbitol, sucrose and maltose, different incubation periods like 24, 48, 72 and 96 hours, pH like 4, 5, 6, 7, 8 and 9 and temperature like 25, 30, 35, 37, 40 and 45°C.

MTT Assay for Lipopeptide on HT-3 cells: The formazan crystals of the blue color produced by MTT dye through mitochondrial dehydrogenase of reduction reaction found in the live cells of mammalian were used to determine the viability of human cervical cancer HT-3 cells. The HT-3 cells were placed in a microplate after being suspended at a final concentration of 1.2×10^4 cells/mL in DMEM with 5% Foetal bovine serum. HT-3 cells were maintained on plates for 24 hours in a CO_2 incubator (37°C; 4% CO_2). The cells were exposed to various levels of the membranes (0.22-micron) of the lipopeptide (5, 15, 25, 35, 45, 55, 65 and 75µg/mL)[37].

The MTT (5mg/mL in $D.H_2O$) was added to the well of a 96-microplate after 24 hours. With the help of an automatic plate reader, the absorption of each well was determined at 570 nm (MULTISCAN EX, Thermo Electron Corporation, China), finally, the cell viability was calculated[38].

Results and Discussion

The bacterial isolates were screened from the soil specimen and at a pH of 7.2, the nutrient broth was used for the isolation. On nutrient agar plates, seven isolated for bacterial colonies were isolated and kept in pure culture and tested fungal strains such as *Aspergillus niger* and *Mucor sp*. From the pour plate, different bacterial isolates were obtained and streaked in a Petri plate. Finally, LP-3 bacterial isolates were identified by using 16S rRNA sequencing. The sequence of actinomycete 16S rRNA was processed and deposited in Genbank (NCBI) with the following accession number OP445738. A strain named *Streptomyces iconiensis* VR1 is shown in figure 1. On potato dextrose agar Petri plates, the fungal species *Aspergillus niger* and *Mucor sp*. were used to assess the antifungal property of the isolated lipopeptide from *Streptomyces iconiensis*. The strongest antifungal activity against *Mucor sp*. was analyzed by the isolated lipopeptide from *Streptomyces iconiensis* (70.8 ± 2.6%).

Figure 1: *Streptomyces iconiensis* VR1 isolated from a soil sample

Impact of nitrogen sources and carbon sources on Lipopeptide production: In the previous study, bacterial species obtained from an oil-contaminated site of a desert location in Gujarat, India was used to produce lipopeptides, which are used as biosurfactants in microbial improved oil recoveries[39]. Similarly, lipopeptide-producing microorganisms were isolated in the polluted regions of the fishing harbor in Nagapattinam, Tamil Nadu[40]. By adding 1% (w/v) of a selected nitrogen source such as ammonium sulphate, ammonium nitrate, peptone, ammonium chloride, beef extract, sodium nitrate, or yeast extract to the production broth, the impact of various nitrogen sources on the production rate of lipopeptide was obtained from *Streptomyces iconiensis*.

Out of six nitrogen sources, beef extracts showed the highest production rate of lipopeptides (1848 mg/L) shown in figure 2a. Similarly, different carbon sources such as glucose, fructose, starch, sorbitol, sucrose and maltose were used in the production broth. Sorbitol showed the highest production rate of lipopeptides (1832 mg/L) shown in figure 2b. Furthermore, with varied pH from 4 to 9 and at pH 7, highest production rate of lipopeptides was obtained at 2956 mg/L shown in figure 2c, whereas in the case of temperature at 30°C, the highest production rate of lipopeptides was obtained at 2209 mg/L shown in figure 2d. Finally, the incubation period was varied from 24, 48, 72 and 96 hours

and enhanced lipopeptide was found to be 720 mg/L at 48 hours as shown in figure 2e, due to the presence of urea in the basal salt medium.

Researchers reported that in the culture medium, if the urea content was retained at 5.01 g/L and glucose as a carbon source (40.0 g/L), the lipopeptide production was up to 750 mg/L[41]. The culture media containing 2.01 g/L of nitrogen sources, 20.00 g/L of carbon sources and 3.00 g/L of dipotassium hydrogen phosphate trihydrate, 10 g/L sodium dihydrogen phosphate, 0.2 g/L magnesium sulphate heptahydrate, 0.002 g/L manganese (II) chloride and 0.2 g/L yeast extracts enhanced the biosurfactant production rate[42]. The greatest C15-Surfactin productivity was obtained for *Bacillus sp.* up to 102.1 mg/L, sucrose as a carbon source and nitrogen source as an ammonium nitrate. After the fermentation, the biosurfactant produced by *Bacillus subtilis* was 720 to 2040 mg/L[43]. The lipopeptide production rate was enhanced in between the range of 1800 -2000 mg/L under optimized media condition pH 7, temperature 30°C, incubation period 48 hours, sorbitol and beef extract at 1% (w/v).

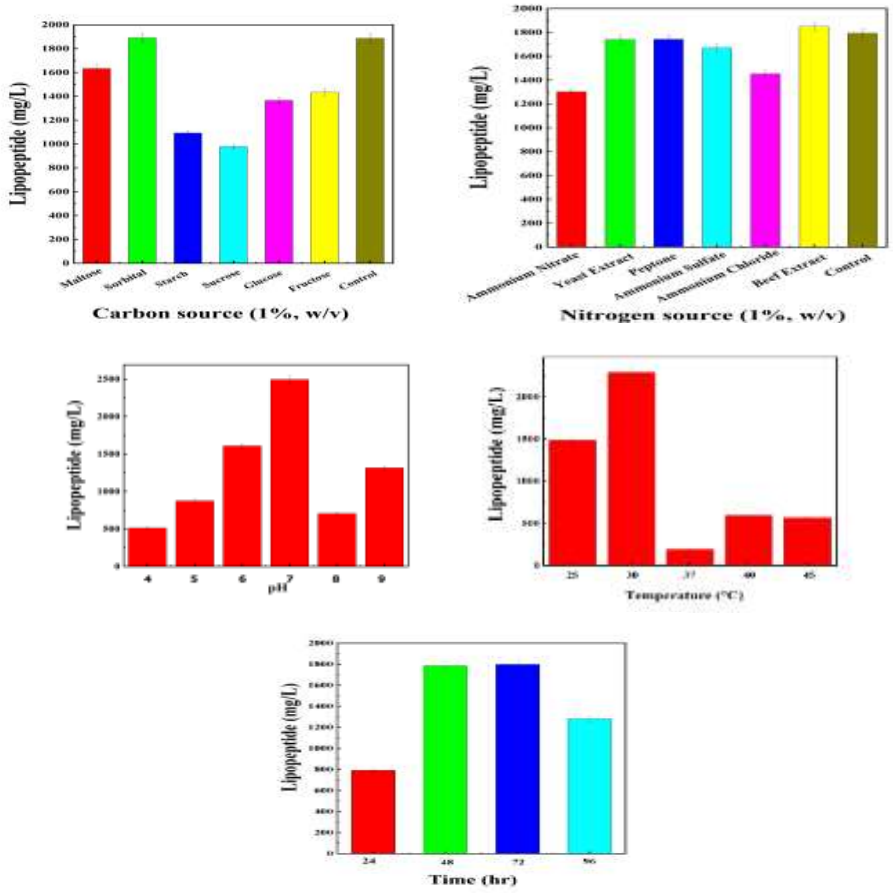

Figure 2: Various nitrogen sources and carbon sources optimization for the productivity of lipopeptides through the *Streptomyces iconiensis*. (a) Optimization of carbon sources (b) optimization of Nitrogen sources; (c) Fermentation broth pH optimization; (d) Temperature optimization and (e) Incubation period time optimization.

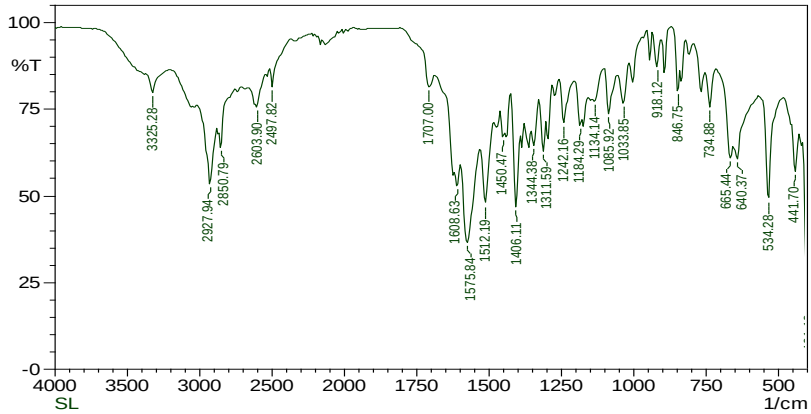

Figure 3: FTIR spectra of the isolated Lipopeptide from *Streptomyces iconiensis*

Table 1
Anticancer activity of lipopeptide isolated from *Streptomyces iconiensis* against HT-3 cells.

Lipopeptide (µg/mL)	HT-3 cell Death (%)
5	45.86± 3.26
15	51.06± 2.44
25	84.22± 2.12
35	91.02± 1.53
45	88.02± 2.43
55	84.81± 3.16
65	86.39 ± 2.43
75	91.24 ± 3.43

The peak at 3225.28 cm^{-1} is due to NH stretching vibrations and the presence of an amine group. The existence of a long aliphatic chain present in the lipopeptide complex is seen at 2927.94 and 2850.79 cm^{-1}. The peak at 1608.63 cm^{-1} showed the presence of an amide functional group containing a carbonyl group. An aromatic group in the lipopeptide shows a peak at 1406.11 cm^{-1} while the C-O bending shows a peak at 1242.16 cm^{-1} as shown in figure 3.

MTT Cell Proliferation Assay: These cells were placed in a microplate and treated with LP (lipopeptide) fractions at different concentrations ranging from 5 to 75 g/mL to determine the growth and reproduction of HT-3 cells. Human cervical HT-3 cancer cells showed the greatest growth suppression (94.68 ± 2.2%) with a 75 g/mL dose of lipopeptide obtained from *Streptomyces iconiensis* as shown in table 1.

Conclusion

This study identifies an appropriate culture medium for lipopeptide produced from *Streptomyces iconiensis VR1* strain isolated from a soil sample. The results show that sorbitol was the most appropriate carbon source compared with glucose, starch, fructose, maltose and sucrose. Similarly, beef extract was found to be a potential nitrogen source compared with other nitrogen sources. The optimized reaction parameter for significant production of lipopeptide was found at 7, temperature 30(°C) and time (72h).

The crude LP extract obtained from *Streptomyces icoiniensis* showed the highest antifungal activity against Mucor sp. with 70.8 ± 2.6%, *Aspergillus niger* 45.3 ± 1.5% of zone inhibition. Consequently, LP extract showed significant cell viability (94.68 ± 2.2%) at 75 g/mL against human cervical HT-3 cancer cells.

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