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ORIGINAL ARTICLE

Isolation, Characterization of *Bacillus amyloliquefaciens* and Screening for Bio-surfactant production

Aliza Parveen[1], Raksha Gupta[1], Sam A Masih[2], Ann Maxton[1*]
[1]Department of Molecular and Cellular Engineering, SHUATS, Allahabad, UP, 211007
[2]Center for Transgenic Studies, SHUATS, Allahabad, UP, 211007
eMAIL: ann.maxton@shiats.edu.in

ABSTRACT
The major problems with chemical surfactants is that they are only slightly biodegradable and they can release toxic chemicals when they decompose and they contribute to the depletion of a non-renewable resource that is highly polluting and may result in skin irritation and allergies. Biosurfactants are derived from natural and biodegradable renewable resources produced by bacteria and are therefore made up of living organisms. During this study, the screening procedure for biosurfactant production from the Bacillus amyloliquefaciens isolated from the soil sample was done so that chemical surfactants can be replaced by biosurfactants extracted from bacterial sources. Soil sample from near oil contaminated area was collected from soil sample of Petrol Pump from Naini area. Targeted B. amyloliquefaciens were grown on common followed by selective media. After proper growth, morphological and biochemical tests were conducted to specify species. Genomic DNA isolation, quantification and 16S rRNA sequencing of B. amyloliuefaciens were done for molecular characterization and sequence were submitted to NCBI with accession number viz., LC 379005.1. Biosurfactant production tests were conducted viz., oil spread assay, emulsification assay, drop collapse test and foaming assay and recorded efficiency of our isolated for successive biosurfactant production from contaminated soil.
Key words: *Biosurfactant, 16s rRNA gene, Bacillus amyloliquefaciens, Oil industry*

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INTRODUCTION

Diverse microorganisms, including bacteria, fungi, and yeasts can biosynthesize biologically surface-active agents called biosurfactants. A large variety of biosurfactants generate by many microorganisms can be sustained in a variety of environments, like soil, desert and marine. Biosurfactants are non-ribosomally synthesized secondary metabolites by actively growing and/or resting microbial cells (bacteria, fungi and yeast) [1]. Based on their chemical composition and microbial origin biosurfactants have been classified into groups, into five major classes which include glycolipids, lipopeptides, phospholipids, neutral lipids and polymeric compounds [2].

Biosurfactants and surface-active compounds synthesized by different microorganisms, attract a pronounced interest owing to their potential advantages over their chemical counterparts. Biosurfactants exhibit a similar or better performance and have less impact on the environment as compared with conventional surfactants, as a result of their higher biodegradability and lower toxicity. Furthermore, biosurfactants are also effective under extreme environmental conditions and can be generated from renewable resources [3]. Due to the above mentioned advantages, biosurfactants have the potential to replace some of the synthetic surfactants used in many fields. Biosurfactants can serve as a potential antimicrobial agent, immunological adjuvant. Biosurfactants have widespread application in food processing application as vital food formulation ingredient. Biosurfactants have widespread application also in cosmetic industry, due to its emulsifying, foaming and water binding capacity that can alter product viscosity and product consistency. Therefore biosurfactants have been proposed to replace chemically synthesized surfactants both in food and cosmetics industry [4]. Biosurfactants in the Oil

Industry are used to accelerate oil recovery due to their potential use in the oil industry with minimum purity and specification, so that the whole cell broth could be used. As compared to chemical surfactants, the biosurfactants are very selective, required in minute quantities, and are also effective under a broad range of oil and reservoir conditions [5].

Gram negative *Bacillus* species are generally biosurfactant producing microorganisms having wide range of applications in many agricultural, pharmaceutical, medical and industrial processes that benefited by their physiological properties and their ability to produce different vital enzymes, antibiotics and metabolites. *Bacillus* species can produce a broad spectrum of lipopeptidebiosurfactants, that are cyclic molecules consisting of a fatty acid of variable length (hydrophobic moiety) linked to a short peptide chain (hydrophilic moiety) of seven or ten amino acids. These lipopeptidebiosurfactants also remain functional under extreme conditions of pH, temperature, and salinity. These compounds additionally exhibit the property of lowering surface tensions, interfacial tensions of liquids.

Lipopeptidebiosurfactants production by *B. licheniformis* and *B. subtilis*are highly potent due to their surface tension reducing ability. Both are spore forming, gram-positive rods. Lichenysin and Surfactinare two well researched biosurfactants produced by *B. licheniformis* and *B. subtilis* respectively.*Bacillus amyloliquefaciens* MD4-12 isolated from oil contaminated soil showed promising ability to produce surfactin.*B. amyloliquefaciens* is able to grow at 30°C under high salinity conditions(NaClconc. 15%) and high alkaline conditions *i.e.,* pH 11 and considered as highest biosurfactant production with higher activity and stability [6]. *B. amyloliquefaciens* was noted to produce highest amylases activity while grown on productive medium possessed 1% starch for 48 h at 50 °C in shaker as batch culture [7].

Keeping the above view in mind we chose the current study with isolation of *B. amyloliquefaciens* followed by morphological, biochemical and molecular characterization and screened its ability for biosurfactant by several assays.

MATERIAL AND METHODS

Collection of soil samples

Microbial samples were collected from soil at Naini Industrial area, Allahabad, Uttar Pradesh with sterilized spatula and stored in plastic bags and were stored at 4°C in ice box.

Isolation of *Bacillus* strains

The above collected rhizosphere soil were air-dried for 3-4 hours and sterilized by dipping plant's roots into sterile saline water and then shake the conical flasks in regular interval for around 30 min [8]. Soil suspension was serially diluted and selected dilutions was inoculated on Luria Broth and streaked as well on LB agar media. The plates were kept for incubation at 37°C for 24 to 72 hrs.

Identification of Amylase Producing Bacteria:

Total five colonies were selected and screened for amylase production by starch hydrolysis assay .Selected isolates were streaked on starch agar plate and kept for incubation for 48 hours at 37°C. Then, iodine solution was poured for 30 sec. on the plate. Occurrence of blue colour around microbial growth showed negative result while clear zone of hydrolysis indicates positive result. Clear zones of hydrolysis producing isolates were considered as amylase producers and investigated further by various different assays.

Characterization of isolates

Cultures were incubated at 35±2°C and identical colonies were taken for further morphological and biochemical studies. Several biochemical tests *viz.* IMVIC, gelatin hydrolysis, Urease test, Arginine dihydrolase test and Carbohydrate fermentation tests were performed for the biochemical characterization of selected rhizobacterial isolates using standard procedures of Bergey's Manual.

Molecular characterization

Total genomic DNA was isolated and quantified at 260/280nm. The 16SrRNA gene was amplified using forward 5'-CCGAATTCGTCGACAACAGA GTTTGATCCTGGCTCAG-3' and primer 5'-CCCGGGATCCAAGCTTACGGC TACCTTGTTACGACTT-3 reverse primers with 35 cycles under standard conditions *i.e.* initial denaturation for 3 min at 94°C, denaturation for 30 sec at 94°C (35 cycles), annealing for 30 sec at 60°C, extension for 1 min at 72°Cand final extension step at 72°C for 7 min duration [9]. Amplified product was purified and sequenced (Applied Biosystems, New Delhi) and submitted to GenBank of NCBI.

Screening for Biosurfactant Production

Oil Spread Assay

Confirmatory oil spread assay was conducted according to established methodology of Morikawa *et al*[10].For this, 20 ml of double distilled water was taken in apetri plate and then 40 µl of crude oil was added on the surface. Total 30 µl of culture (cell free) broth was spread over the oil surface. Oil will be

displaced, if biosurfactantis available in culture broth, Clear zone with no oil shows surfactant activity, also called as oil displacement activity. During this assay, a control was maintained without surfactant that was not contained any clear zone.

Emulsification test

Emulsification activity for bacterial isolate was calculated on the basis of emulsification index(E_{24}). Emulsification test was performed by taking 2ml of oil in 1ml supernatant (cell free), and then vortexed for 4-5 minutes confirming proper mixing of both liquids. Emulsification activity was calculated after 24 hours duration using below mentioned formula:

$$E_{24} = \text{Total height of the emulsion layer/height of the aqueous layer} * 100$$

Drop Collapsing Assay

Biosurfactant production by *Bacillus amyloliquefaciens* was conducted by qualitative test *i.e*., drop-collapse assay that relies on destabilization of liquid droplets through surfactants. For this, drops of culture supernatant or cell suspension were spread overoil coated solid surface. If culture does not possess surfactants, the polar water molecules are repelled from the hydrophobic surface and drops remain stable. If culture holds surfactants, drop collapse evenly or spread because of interfacial tension or force between liquid particles and reduction in hydrophobic surface can be observed. Stability of drops is depending on surfactant concentration while correlates with interfacial tension and surface. Bacterial culture was spread over oil-coated regions to observe any developed drop size after 50-60 sec. Flat drop shows positive while round drop shows negative results and indicates lack of biosurfactant production.

Foaming Assay

Isolated strain was kept separately in a flask with 100 mL nutrient broth medium and incubated for 72 h duration at 37°C on a shaker. Foaming activity was determined as foam height, shape and total duration of foam stability in the graduated cylinder.

RESULTS AND DISCUSSIONS

Isolation of *Bacillus spp*. from soil sample

In the present study the soil sample was incubated for 15 min at 80°C before the serial dilution for the selective isolation of bacillus species. Many Colonies (five) of *Bacillus spp.* were isolated from the soil sample as plates were incubated at 37±2°C and developed colonies were identified on the basis of cultural, morphological as described in the Bergey's Manual of Determinative Bacteriology (Table 1). Some scientists have successfully isolated *Bacillus spp*. by incubating the soil sample at 80°C for 10 minutes [8].

Table 1: Morphological and Biochemical characteristics of isolates

Morphological Characters	Test	Results
	Colony shape	Irregular
	Margin	Lobate
	Colony elevation	Raised
	Colour	Creamy Dull
	Texture	Dry (Rough)
Stainng	Gram Staining	Positive
Biochemical Characters	Indole	Negative
	Methyl Red	Negative
	Voges-Proskauer	Negative
	Urease	Negative
	Gelatin hydrolysis	Positive
	Arginine dihydrolase	Negative

Identification of Amylase producing bacteria:

All the five samples *i.e.* A, B, C, D and E were tested for amylase production in which the isolate B showed a negative result with no amylase production for starch degradation while all other A,C, D and E isolates showed positive results for amylase test (Fig. 1).

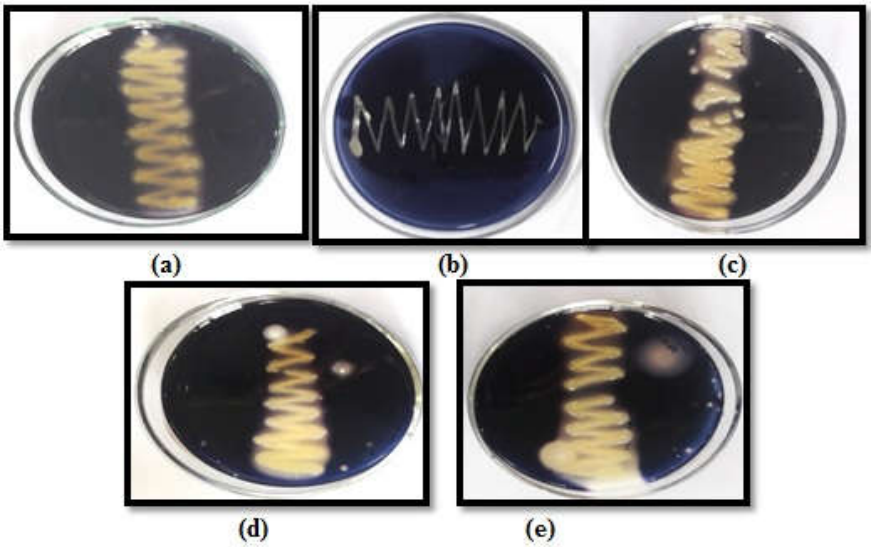

Fig. 1:Amylase Producing *Bacillus sp.* (five isolates)**.**

According to the previous study *B. amyloliquefaciens shows an*increased production of α-amylase [11].

Biochemical Characterization

There was no visualization of cherry red reagent layer inany of the tubes, hence, the samples showed a negative indole test which indicated that they cannot oxidize tryptophan (Table 1).In a study the various biochemical reactions the *B. amyloliquefaciensgavea negative result for indole test and was unable to oxidize tryptone* [12]. In this test all the four isolates were found to produce a positive test and were able to liquefy gelatin (Table 1).

In a study, total 1075 *Bacillus* strains were expressed as positive for each species and confirm *B. amyloliquefaciensgavea positive result for gelatin test* [13]. In VP test, two isolates gave the positive test which were able to produce acetoin; 2,3 butanediol and ethanol which causes less lowering of pH while two isolates gave negative test (Table 1). During Methyl Red test, two isolates gave the positive test which was producing acid by mechanism of mixed acid fermentation from glucose and two isolates were giving a negative test (Table 1).

Urease test for all four isolates were found to be a negative test as they were unable to hydrolyse urea and there was no colour change were observed after inoculation and incubation (Table 1).The isolate which was *Bacillus amyloliquefaciens* gave a negative result for the urease test as it was not able to hydrolyse it [13].

In arginine dihydrolase test all the isolates gave a negative test as they were unable to hydrolyze arginine to ornithine. There is no change in the pH and hence, the colour of the media remains unchanged (Table 1).

Carbohydrate fermentation test:

A positive carbohydrate fermentation test shows colour change (red to yellow). The isolates gave the following tests (galactose, lactose, maltose and manitol) for different sugars and for all tests isolates A and D showed positive results (Fig. 2).

In a study the results of the tests for the 1075 *Bacillus* strains were expressed as percentages of positive results for each species or group of strains and hence *the different strains of Bacillusgave different results for the different carbohydrate*[12]**.**In the current study the different carbohydrate tests gave the desired result for the isolate A.

Molecular Characterization:

After confirming morphological and biochemical characterization, total genomic DNA was extracted and run on agarose gel with 1kb ladder. Bacterial culture showed genomic DNA size greater than 10kb size Total genomic DNA from *Bacillus* species was isolated and screened for quantification using UV spectrophotometer at OD 260 and 280 nm to calculate total DNA conc. (µg/ml). Total genomic DNA conc. was recorded 320µg/ml while 1.83 was observed as ratio. After successful qualification, genomic DNA was run in 1.2% agarose gel for 23 min at 75 V. Product of genomic DNA was amplified using forward and reverse primers to screen 16s rRNA gene of *Bacillus* species with 1 kb ladder (Fig. 3). Amplified product was then sequenced for 16S rRNA at Applied Biosystems using the oligonucleotide primers and after sequencing, bacterial isolate sequence was submitted to DNA Database Bank of Japan (DDBJ), a

collaborator of NCBI and obtained accession number *i.e.*,LC379005.1 and species was confirmed and *Bacillus amyloliquefaciens* with 1468bp size. Using these amplified sequences a BLAST search was performed where a similarity in marked sequence was observed in the NCBI GenBank. The phylogenetic analysis of these 16S rRNA partial sequences of selected two isolates with previously available sequences in NCBI database confirmed significant polymorphism among these sequences. The phylogenetic tree generated revealed that sequences of *B. amyloliquefaciens* fell into the same clade with the same genus.

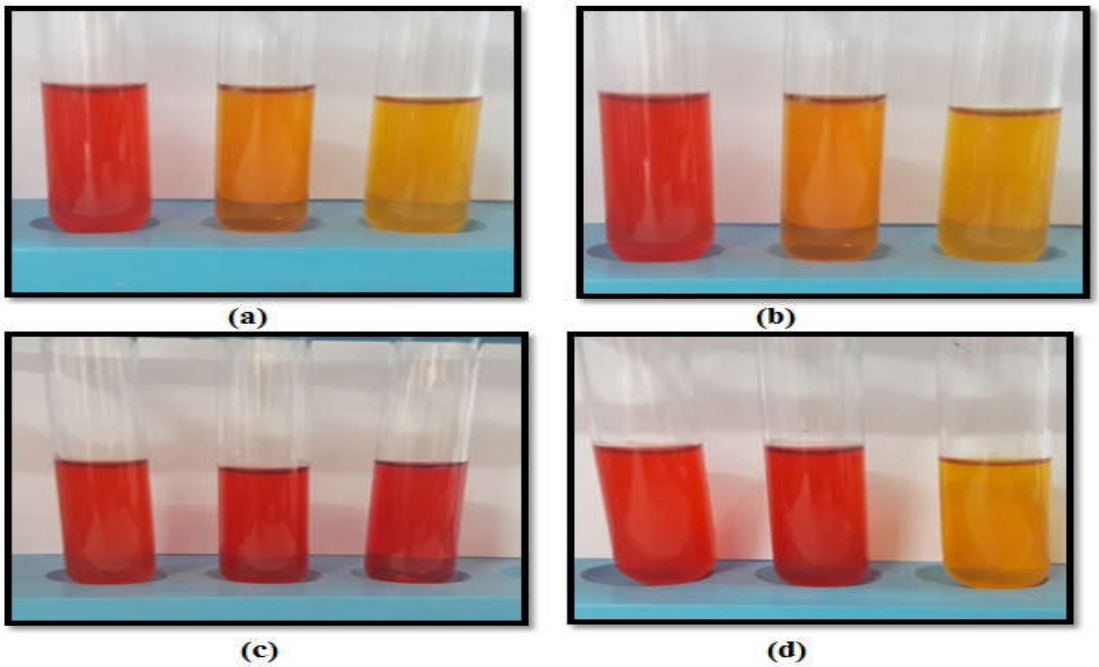

Fig. 2: Carbohydrate fermentation test for (a) galactose, (b) lactose, (c) maltose and (d) mannitol with *Bacillus spp*.

Fig. 3: 16s rRNA gene amplification for isolates (Lane 1: 1kb Ladder, Lane 3:*B.amyloliquefaciens*)

Screening for bio-surfactant production

Oil Spread Assay

Biosurfactant activity was present in isolate culture broth as oil was displaced and clear oil free zone and diameter (30cm) showed surfactant or oil displacement activity while control could not produce any clear zone (Fig. 4.) was observed that showed positive test for our test and ability to bio-surfactant production. In a study the investigators used oil spread assay for screening of biosurfactant. As a result there was production of a clear zone in the maximum level [14].

Emulsification assay

The isolated *B.amyloliquefaciencs* strain has shown positive result while checkedfor its ability to emulsify crude oil. Test was conducted by keeping 1 ml of supernatant with 2ml crude oil overnight. Emulsified layer was measured as 0.7 cm while total liquid layer was noted as 1.8 cm and E_{24} (%) was 38.88 that

again confirmed ability of our isolates for bio-surfactant production (Fig. 5)In another study for the screening of bio-surfactant producing microorganism; emulsification assay of the organism which was under trial showed the highest production of bio-surfactant [14].

Drop collapsing test

The result of the drop collapsing assay was positive since the drop containing bio-surfactant producing culture was collapsed and became flat instead of round due to the or interfacial tension or force between culture liquid drop resulting reduction in hydrophobic surface (Fig. 6). For investigation in the drop collapse method the three organisms in test were collapsed. This indicated that the three organisms were positive for biosurfactant production [14].

Foaming assay

Results were noticed after 72 h duration for *B.amyloliquefaciencs* strain and sufficient foam was noticed (Fig. 7) that showed positive results for foaming assay and confirmed ability *B. amyloliquefaciens* for bio-surfactant production [14].

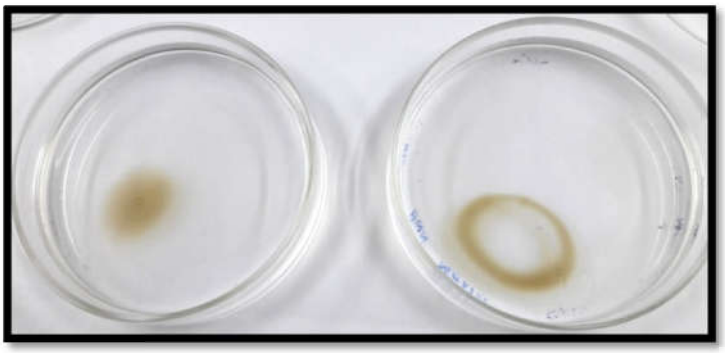

Fig. 4: Oil spread assay

Fig. 5: Emulsification assay

Fig. 6: Drop collapsing assay

Fig. 7: Foaming assay

CONCLUSION

Biosurfactants, surface-active compounds are fetching pronounced interest since they holds potential advantages over several chemical compounds as they possess better output with less negative impact on exposed environment since they have high level of biodegradability with lower toxicity. Till date several works are going on to isolate different biosurfactants from different microbial strains that have ability to produce biosurfactants. In our experiment *Bacillus amyloliquefaciens* was isolated, characterized and screened for biosurfactant production by various screening techniques (Drop collapse test, Emulsification assay, Heamolytic activity, Oil spread assay and Foaming assay) which gave the positive results for production potential of *Bacillus amyloliquefaciens*. In heamolytic activity a clear zone was observed on plates while duringOil displacement with oil free clearing zones showed the positive result and clearing zone diameteremphases surfactant activity of isolated B. *amyloliquefaciens*LC379005.1.Confirmatory Drop collapse assay signified the biosurfactant activity by spread or collapse of drop on solid media coated with oil. Future prospects of present study further screening and extraction studies from the oil contaminated soil and water bodies and increment in potential of producing biosurfactant.

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