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Research Article

PRODUCTION OF BIOSURFACTANT FROM DATES AND ITS ACTIVITY AGAINST BIOFILM PRODUCED BY *CANDIDA* AND *E. COLI*

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Abstract- The aim of this research was production of biosurfactant from the normal flora of Dates (*Phoenix dactylifera*) to check its activity against biofilm produced by *Candida* and *E.coli*. The organisms were isolated LBD (Lactic acid bacteria differential agar) and the plates were incubated at room temperature for 48 hours. Several biochemical test were performed with this strains. Drop collapse test was performed and was found to be +ve. The organism was inoculated in MSM broth (mineral salt medium) with petrol as a source of carbon for biosurfactant production and organism was found to be degraded in MSM broth. Biosurfactant was extracted and purified in ethyl acetate and methanol (4:1). The biosurfactant extracted was checked against biofilm formed by *Candida* in catheter and *E.coli* in IV set and was incubated overnight. After incubation catheter and IV set was washed 3 times with saline. For checking the biosurfactant activity Catheter and IV set was dipped in saline with biosurfactant by cutting them into small pieces. They were incubated overnight and Spread plate was performed for each wash and variation in growth was observed in gradient form. The organism was characterised at NFB by 16s RNA sequencing method and was found to be *Acinetobacter pittii*.

Keywords- *Biosurfactant, Dates, Biofilm*

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Introduction

Biosurfactants produced from bacteria which is been isolated from a natural product such as date with petrol as a source of carbon. The definition of biosurfactants and microbes involved in the production, physiology and genetic regulation of biosurfactant. Advantages disadvantages of use of biosurfactants and application of biosurfactants in industries and its medical importance are discussed in detail. International, National and regional scenarios of biosurfactant production and utilization is also presented in the chapter. As well as its activity against biofilm produced by *Candida* and *E. coli*. Biosurfactants are diverse group of surface-active molecules /chemical compound synthesized by microorganisms. These amphiphilic compounds are produced on living surfaces, mostly on microbial cell compounds are produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly. These are amphipathic molecules having both hydrophilic and hydrophobic domains that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tensions at the surface and interface respectively. Most biosurfactants are either anionic or neutral and the hydrophilic moiety can be a carbohydrate, an amino acid, a phosphate group, or some other compounds.

Classification and chemical nature of biosurfactants

Chemically classified surfactants are usually classified according to their nature of polar groups but biosurfactants are generally categorized by their chemical composition dictated by different molecules forming hydrophilic and hydrophobic moieties and microbial origin. The hydrophilic moieties may consist of amino acids, peptides, mono, di, polysaccharides. The hydrophobic moieties may consist of saturated and unsaturated fatty acids suggests that biosurfactants can be classified in low-molecular-mass-molecule, which efficiently lower surface and interfacial tension, and high molecular mass polymers, which are not efficient as emulsion stabilizing agents.

Genetic biosynthesis and regulation of biosurfactant production

Biosurfactants display a range of different amphiphilic structures. Biosurfactants are made up of hydrophobic and hydrophilic moieties. For synthesis of these two moieties two different synthetic pathways must be used: one leading to the hydrophobic and one to the hydrophilic moiety. The hydrophobic fatty acid components- which may be a long chain fatty acid, a hydroxyl fatty acid or alpha-alkyl-beta-hydroxy fatty acid are synthesized by rather common pathway of lipid metabolism.

Among all the biosurfactants reported till date, the molecular biosynthetic regulation of rhamnolipid, a glycolipid type biosurfactant produced by *Pseudomonas aeruginosa* and a lipopeptide biosurfactant called surfactin produced by Bacillus subtilis were the first to be deciphered. Other biosurfactants whose molecular genetics have been delineated in the recent years include arthrofactin from *Pseudomonas* sp., iturin and lichenysin from *Bacillus* species, mannosylerythritol lipids (MEL) from *Candida* and emulsan from Acinetobacter species. A putative rhamnolipid biosynthesis pathway is summarized.

The biosynthetic pathway can be divided into three major steps; synthesis of the hydrophilic part, synthesis of the hydrophobic part and synthesis of rhamnolipid from these two parts. The precursors, dTDP-L-rhamnose and activated 3-(3-hydroxyalkanoyloxy) alkanoate (HAA) respectively for hydrophilic and hydrophobic parts are synthesized *de novo*. Altogether the biosynthesis can be separated into three major parts. Finally, the rhamnolipid is produced by the reaction of two special rhamnosyl transferases catalyzing the sequential rhamnosyl transfer reactions from the precursors over mono- toward di- rhamnolipids.

In *Pseudomonas aeruginosa*, several genes have been found to be involved in rhamnolipids biosynthesis. Ochsnen discovered a 2-kb fragment capable of restoring rhamnolipid biosynthesis while tested in a rhamnolipid deficient mutant strain of *Pseudomonas aeruginosa*.

The 2-kb fragment contains a single open reading frame (*rhlR*) of 723bp specifying a putative 28-kDaprotein (*RhlR*). Disruption of the *Pseudomonas aeruginosa* wild-type *rhlR* locus led to rhamnolipid- deficiency, thus confirming directly that this gene is necessary for rhamnolipid biosynthesis. The *rhlAB* genes encode a rhamnosyl transferase, *RhlAB*, which catalyzes the transfer of rhamnose from TDP-rhamnose to β- hydroxydecanoyl-β-hydroxydecanoate. The transcriptional activation of *rhlAB* appears to depend on a functional *RhlR* regulatory protein. The sequence upstream of the *rhlA* promoter contains two inverted repeats that define putative binding sites for the *RhlR* regulator. Another gene, *rhlI*, which is also required for rhamnolipid synthesis, has been identified downstream of the *rhlAB*R gene cluster. The *rhlI* gene production, *RhlI*, has been proved to be a bacterial autoinducer (usually belongs to homoserine lactone family) synthase. The *Pseudomonas aeruginosa rhlA* promoter is active only when both the *rhlR* and *rhlI* genes are present or when the *rhlR* gene alone is supplied together with synthetic autoinducers. The *RhlR-RhlI* regulatory mechanism is known as quorum sensing (QC). QC describes population density dependent cell to cell communication in bacteria using diffusible signal molecules. These signal molecules produced by bacterial cells, regulate various physiological processes important for social behaviour and pathogenesis like synthesis of rhamnolipid in *Pseudomonas aeruginosa*. Environmental factors influence the synthesis of biosurfactant

Synthesis of biosurfactant like any other chemical reaction is influenced by a number of environmental factors that either increase its productivity or inhibit it. Literature shows that different environmental factors are required for synthesis of biosurfactant by different microbial sp. Same conditions are not suitable for all the microbes. For example, some bacteria synthesize maximum biosurfactant in hexadecane whereas some others cannot tolerate n-hexadecane. Environmental factors such as pH, temperature, salinity, agitation and oxygen supply affect biosurfactant production. The type, quality and quantity of biosurfactant produced are influenced by the nature of the carbon substrate, the concentration of N, P, Mg, Fe, and Mn ions in the medium, and the culture conditions. However, it was reported that biosurfactant production from *Pseudomonas* strains MEOR171 and MEOR172 are not affected by temperature, pH, and Ca, Mg, concentration in the ranges found in many oil reservoirs. Interestingly, recently proposed that P. aeruginosa is producing rhamnolipids to reduce oxygen transfer rate as a means to protect itself from oxidative stress, and it appears that this mechanism is activated by iron deficiency

Procedure

Isolation on LBD agar

1. Take 5gm of dates sample and dissolve in sterile saline.
2. Store it overnight
3. Take a loopful of saline suspension and streak it on LBD agar and incubate it for 48 hours at room temperature.
4. Colonies appeared.

Isolation Rogosa agar

5. Take a loopful of saline suspension prepared earlier and streak it on rogosa plate.
6. Incubate for 48 hours at room temperature
7. Colonies appeared
8. Perform gram staining
9. Pickup a bacterial colony and add it in sterile saline
10. Half the suspension in another sterile test tube and keep one for UV exposure and other at Room temperature.
11. Inoculation in MSM Medium
12. Prepare 2 flasks of MSM medium in addition with 5-6 drops of petrol. Place culture suspension which is untreated in one flask of MSM and UV treated bacterial suspension in another flask for 4-5 days at room temperature. Cover the flask which is being inoculated with uv treated culture suspension with black paper.
13. Adjust the pH of msm medium by acidifying it at 2 pH with 6N HCL storing it at overnight at 4 degree Celsius
14. For extraction of biosurfactant the culture inoculated MSM medium was washed three times with ethyl acetate and methanol (4:1). An organic layer was formed which was been pipette out in sterile petri plate. Organic layer was evaporated at room temperature for 24 hours. Acetone was added for biosurfactant recovery.

For Activity Against Biofilm

Culture suspension of *Candida* and *E.coli* was allowed to run in Catheter and glucose saline pipe Incubated at RT for 24 hours

The culture inoculated catheter and glucose saline pipe were cut in pieces of 5 cm and inoculated in sterile saline. Of which one piece of catheter and glucose pipe was washed one-time, other piece two times and another 3 times with saline by making dilutions of 10-1, 10-2, 10-3.

Now the culture inoculated pieces of catheter and glucose pipe were placed in 5 ml saline and few drops were added in each of the tube saline suspension.

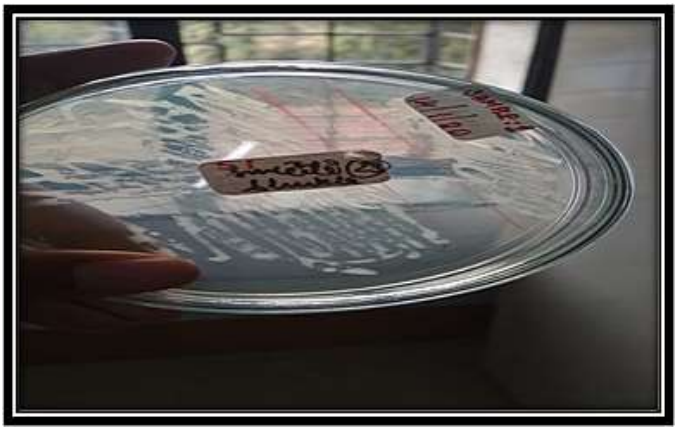
Incubate at RT for 24 hours

For checking the activity of biosurfactant plates spread plate was performed and also the suspensions with biosurfactant were agitated and spread plate was also performed for them. Biosurfactant produced from organism that was UV treated was found to be mutated and this was concluded by non-inhibiting activity against *candida* and *E. coli* biofilm. Organism was sent for characterization at NFB and was found to be *Acinetobacter pittii*.

Result

The present result is focused on biosurfactant production and its activity against biofilm produced by *Candida* and *E.coli*. Biosurfactant was produced from dates samples and petrol as a source of carbon. The result was carried out by isolating organisms on LBD and Rogosa medium. Round colonies were observed and these colonies were subjected to biochemical test and gram staining.

Dates sample added in sterile saline for making sample suspension A loopful of saline suspension was streaked on LBD as well as Rogosa agar and incubated for 48 hours and bacterial colonies appeared.



Gram staining of the culture

Simmon Citrate test was performed and as the slants colour changed from blue to bluish green indicating the organism is citrate positive

Urease test was performed and the color of the slant remain unchanged indicating the organism is urea negative.

Catalase test was also performed and a slight effervescence was observed indicating the organism may be catalase positive.

The bacterial suspension was divided in two sets one was without uv treated and other was exposed to UV.

The pH of MSM was adjusted at 2 with 6N HCL and refrigerated overnight after that organism was inoculated in MSM and5-6 drops of petrol as a source of carbon and incubated for 4-5 days.

After incubation extraction was carried out by washing the MSM broth containing organism with Ethyl acetate and Methanol (4:1) for three times an organic layer was formed over the surface of MSM media.

After extraction methanol was evaporated and biosurfactant was dissolved in acetone and evaporated

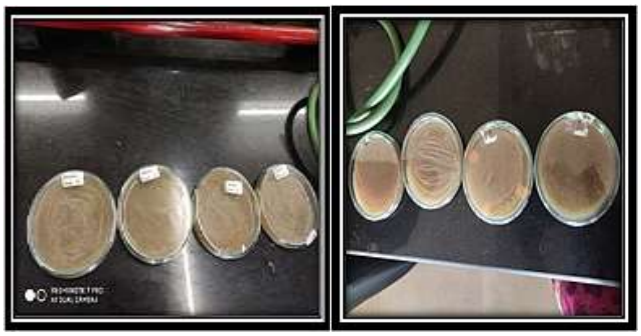
Suspensions of *Candida* and *E. coli* were run in Catheter and IV were incubated at refrigerator overnight.

Drop collapse test was performed by using 5ml of engine oil on a petriplate and pouring 2 drops of biosurfactant over the engine oil.

Catheter and IV set were washed 3 times simultaneously and were cut into pieces of 5 cm each.

This piece of catheter and IV set were inoculated with biosurfactant in sterile saline and incubated overnight at RT for 24 hours. Spread plate was performed of the suspension with biosurfactant agitated and without agitated.

Candida + biosurfactant



Non agitated Agitated

E. coli + biosurfactant

Non agitated Agitated

The organism was characterized at NFB by 16s RNA sequencing method and was concluded as *Acinetobacter pittii* strain HPPD02

ACGGCTGGCAGGCCAGGAATAAACTACAATGTAAGTGCGAGTAGGCGCGGA
AGAGTGCTTTGTCTCTATGTGATTAAGCGGCCGGACGGGGTGAAGTAATGGT
TTGGGAATACTGCCCTAGATGGGAGGGGGACTAACATATTGAAAAGGAATGG
TTTATTCCCGCCTTACGTCTCTACAGGAGAAAGCAGGGGGATCTTTGGGACC
TTGATGTAATAGAAGATCCCAAGTTGGATTAGGTTGTTGGTGGGGTAAAGGC
CTAACATGGGGGAGATTCCTAGGGGGTTTGAGAAGAAGGTCCGCCACAATG
GGAATGAGACCAGGCCCCGAATTCTAAGGGAGGCCGCCATGGGGAATTTTG
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TTTGGTTGTAAAGCAATTTTAGGGAGGGGGGAAGTAGTTAAGTTAATACACAG
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CCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGC
GCACGTAGGCGGTTAATTAAGTCAAATGTGAAATCCCCGAGCTCAACTTGGG
AATTGCATTCGATACTGGCTAGCTAGAGTGTGGTAGAGGATGGTAGAATTCC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGG
CAGCCATCTGGCCTAACACTGACGCTCAGGTGCGAAAGCATGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTATTAGCAGGT
GGTGGCCTTTGAGGCTTTACTTGCCGCAGCTAACGCGATAAAGTAGACCGCC
TGGAGA

Phylogenetic Tree(Origin)

1 acggctggca ggccaggaat aaactacaat gtaagtgcga gtaggcgcgg aagagtgctt
61 tgtctctatg tgattaagcg gccggacggg gtgaagtaat ggtttgggaa tactgcccta
121 gatgggaggg ggactaacat attgaaaagg aatggtttat tcccgcctta cgtctctaca
181 ggagaaagca gggggatctt tgggaccttg atgtaataga agatcccaag ttggattagg
241 ttgttggtgg ggtaaaggcc taacatgggg gagattccta gggggtttga gaagaaggtc
301 cgccacaatg ggaatgagac caggccccga attctaaggg aggccgccat ggggaatttt
361 ggacaaaggg ggccagcctg atcccaccct gccgggtgtt agaagaaggc cttttggttg
421 taaagcaatt ttagggaggg gggaagtagt taagttaata cacagagata atggacgtta
481 ctcgcagaat aagcaccggc taactctgtg ccagcagccg cggtaatacagagggtgcaa
541 gcgttaatcg gatttactgg gcgtaaagcg cacgtaggcg gttaattaag tcaaatgtga
601 aatccccgag ctcaacttgg gaattgcatt cgatactggc tagctagagt gtggtagagg
661 atggtagaat tccaggtgta gcggtgaaat gcgtagagat ctggaggaat accgatggcg
721 aaggcagcca tctggcctaa cactgacgct caggtgcgaa agcatgggggcaaacagga
781 ttagataccc tggtagtcca tgccgtaaac gatgtctatt agcaggtggt ggcctttgag
841 gctttacttg ccgcagctaa cgcgataaag tagaccgcct ggaga

Conclusion

The main aim of this research work was to carry out production of biosurfactant by isolating biosurfactant producing bacteria from dates. The organism was isolated on LBD and Rogosa agar and inoculated in MSM broth. The organism was found to be citrate positive, catalase positive, urease negative, oxidase negative and indole negative. Gram staining was performed and organism appeared as pink rods indicating its gram negative. The bacteria were identified by 16s RNA sequencing method by NFB and was concluded as *Acinetobacter pittii*.

> *Acinetobacter pittii* Strain HPPD02 16S ribosomal RNA gene, partial sequence

ACGGCTGGCAGGCCAGGAATAAACTACAATGTAAGTGCGAGTAGGCGCGGA
AGAGTGCTTTGTCTCTATGTGATTAAGCGGCCGGACGGGGTGAAGTAATGGT
TTGGGAATACTGCCCTAGATGGGAGGGGGACTAACATATTGAAAAGGAATGG
TTTATTCCCGCCTTACGTCTCTACAGGAGAAAGCAGGGGGATCTTTGGGACC
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AGATAATGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAG
CCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGC
GCACGTAGGCGGTTAATTAAGTCAAATGTGAAATCCCCGAGCTCAACTTGGG
AATTGCATTCGATACTGGCTAGCTAGAGTGTGGTAGAGGATGGTAGAATTCC
AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGG
CAGCCATCTGGCCTAACACTGACGCTCAGGTGCGAAAGCATGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTATTAGCAGGT
GGTGGCCTTTGAGGCTTTACTTGCCGCAGCTAACGCGATAAAGTAGACCGCT
GGAGA

Phylogenetic Tree (Origin)

241 ttgttggtgg ggtaaaggcc taacatgggg gagattccta gggggtttga gaagaaggtc
301 cgccacaatg ggaatgagac caggccccga attctaaggg aggccgccatggggaatttt
361 ggacaaaggg ggccagcctg atcccaccct gccgggtgtt agaagaaggc cttttggttg
421 taaagcaatt ttagggaggg gggaagtagt taagttaata cacagagata atggacgtta
481 ctcgcagaat aagcaccggc taactctgtg ccagcagccg cggtaatacagagggtgcaa
541 gcgttaatcg gatttactgg gcgtaaagcg cacgtaggcg gttaattaag tcaaatgtga
601 aatccccgag ctcaacttgg gaattgcatt cgatactggc tagctagagt gtggtagagg
661 atggtagaat tccaggtgta gcggtgaaat gcgtagagat ctggaggaat accgatggcg
721 aaggcagcca tctggcctaa cactgacgct caggtgcgaa agcatgggggcaaacagga
781 ttagataccc tggtagtcca tgccgtaaac gatgtctatt agcaggtggt ggcctttgag
841 gctttacttg ccgcagctaa cgcgataaag tagaccgcct ggaga

Future Prospective

Biosurfactants have recently become important products of biotechnology and medical applications. Producing biosurfactants from dates and petrol as a carbon source is an economical method.

Biosurfactants are biodegradable and do not pollute environment in any way since chemically synthesized surfactants are non-biodegradable and pollute environment. Also, biosurfactants have an inhibitory response to biofilms produced by species such as *Candida*, *E. coli*, *etc.* So biosurfactants have a rival response to this infection causing biofilms.

Application of research: Production of biosurfactant from dates and petrol does not eliminate any waste material and is economical method.

Research Category: Biotechnology

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****Research Guide or Chairperson of research: Dr Heena Asif Pathan**
University: University of Mumbai, Mumbai, Maharashtra, India
Research project name or number: MSc Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Grocery shop near Jogeshwari, Mumbai

Cultivar / Variety / Breed name: Dates

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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