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Bacterium Isolated from Coffee Waste Pulp Biosorps Lead: Investigation of Biosurfactant Mechanism to Bioremediate Lead Pollution

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Abstract

Klebsiellapneumoniae Kpn555, isolated from coffee waste pulp showed high level of tolerance to lead with a minimum inhibitory concentration of 900 mg/L. On its growth in nutrient broth supplemented with lead, brown clumps were visualised at the bottom of the flask. On scanning and transmission electron microscopic studies the brown clumps were corroborated to be bacterial cells with lead biosorbed on the cell surface and accumulated inside the cytoplasm. Isolation of plasmid from *K. pneumoniae* Kpn555 revealed the presence of a plasmid of size 30-40 kb. This capability of the bacteria was proven to be plasmid mediated as the Escherichia coli DH5α cells transformed with the plasmid of K. pneumoniae Kpn555 also could tolerate 900 mg/L of lead and form brown clumps. The heavy metal bioremediation activity of the produced biosurfactant was also investigated. The biosurfactant was subjected to biochemical characterisation as well as FTIR analysis to establish the chemical nature of the produced molecule. The highest metal removal rate using the biosurfactant was found to be 50% at 400 ppm of Pb. This study shows that this bacterium serves as an effective agent for the removal of lead from contaminated sites.

Keywords: *K. pneumonia* Kpn555, lead, plasmid, biosorption, transformation

Introduction

Contamination of heavy metals is a major concern because of their toxicity and threat to living forms and environment [1]. Due to global industrialization and nuclear processes, large amounts of heavy metals have been released into the biosphere. Among the different heavy metals like copper, cadmium, arsenic, mercury etc, pollution by lead is worldwide public problem, because it causes serious health hazards in children like permanent brain damage, learning disabilities, hearing losses and behavioral abnormalities whereas in adults, it is known to cause hypertension and heart diseases [2]. Half-life of lead in blood is about 1 month and in the skeleton 20-30 years [3]. The removal of heavy metals from the environment is being done by physico-chemical methods such as mechanical screening, hydrodynamic classification, gravity concentration, adsorption, ion exchange, membrane filtration, electro dialysis, reverse osmosis, ultra filtration and photo catalysis [4]. These methods have many disadvantages such as high cost, non-specificity, low efficiency, incapability to cause complete mineralization and therefore they are not suitable for large scale use [5]. Hence, the present day challenge is to develop a novel cost effective environmental friendly method to remove lead from the environment.

In the presence of high concentration of toxic metal ions, microorganisms employ different detoxification processes such as oxidation/reduction of metal ions, formation of complex/precipitates, bioaccumulation/biosorptionetc [6]. The ability of the microorganisms to detoxify metals can be natural or acquired through plasmids. The occurrence of plasmid in metal tolerant strains is often observed in a polluted site rather than an unpolluted site [7]. Biosorption is a process that occurs naturally in microorganisms, which allows them to concentrate and bind contaminants onto their surface or inside their cellular structures. This process could be used as an effective method to remove heavy metal contaminants from the environment that offers several advantages like stability over a wide pH and temperature which is cheaper and also minimizes waste created by the detoxification process. Even though there are plenty of studies on bioaccumulation of metal ions by bacteria [8,9], there are limited reports on the mechanism of bioaccumulation.

Application of biosurfactant in heavy metal remediation is one among the recent, eco-friendly techniques. Biosurfactants are surface active compounds produced by various microorganisms like bacteria and fungi as secondary metabolites, which possess both hydrophilic and hydrophobic moieties. Structurally, they possess a hydrophobic moiety comprising of saturated or unsaturated fatty acids or hydrocarbon chains and a hydrophilic moiety of peptide cations or anions, mono-, di or polysaccharides.

Several bacterial isolates belonging to the genus Bacillus, Pseudomonas, Arthrobacter, Micrococcus,andRahnella have been reported as efficient BS producers. JN6 effectively improves the efficiency of phytoremediation in soils contaminated by Cd, Pb, and Zn. The biosurfactant produced by Acinetobacter sp. has been reported to bioremove up to 14.04% of Pb while that produced by Pseudomonas putida T1 has been reported to remove up to 6.5% and 2.01% of Zn and Cu respectively.

In this study, the lead tolerance of Klebsiellapneumoniae Kpn555, isolated from coffee pulp waste was tested. Growth of bacteria in response to various concentrations of lead was studied and scanning electron microscopy (SEM), transmission electron microscopy

(TEM) and energy dispersive spectrometry (EDS) analysis was employed to investigate the mechanism behind removal of lead. In order to examine if the heavy metal resistance was genome or plasmid mediated, plasmid isolation and genetic transformation studies were also employed.

Materials and Methods

Microorganism and culture maintenance

The strain used in this study was isolated from coffee waste pulp and

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identified by 16s rDNA sequencing as Klebsiellapneumoniae Kpn555. The gene sequence of this strain is deposited in NCBI with accession number KX570899.1. The strain was stored as glycerol stocks at -20°C and subcultured periodically.

Determination of minimum inhibitory concentration (MIC) of lead

Nutrient Broth (NB) (Hi-Media Lab. Ltd., Mumbai, India) was prepared in test tubes and supplemented with different amounts of lead to obtain the desired concentration ranging from 100-1500 mg/L. These tubes were inoculated with 1% v/v of 16 h old culture of K. pneumoniae (1 × 106 cells) and incubated for 24 h at 37°C. The growth of K. pneumoniae Kpn555 in this medium was analyzed by measuring the Optical Density (OD) at 600 nm on a UV-visible spectrophotometer (Shimadzu, Japan). MIC was defined as the minimum inhibitory concentration of the heavy metal that showed an OD of 0.1 at 600 nm after 24 h of incubation.

Growth kinetics and biosorption of lead

NB (100 ml) was supplemented with different concentrations of lead (100 mg/L, 400 mg/L and 800 mg/L) based on MIC results and inoculated with 1%v/v of 16 h old culture(1 × 106 cells). The OD of the broth was measured at 600 nm periodically and the growth kinetics was plotted. The cell free supernatants were analysed for residual lead concentration on an atomic absorption spectrophotometer (AAS-GBS-AVANTA).

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive spectrometry (EDS) analysis

Brown clumps, settled at the bottom of the culture medium were analysed by observing them under SEM (Hitachi S4100, Japan) and TEM. For SEM analysis, the clumps were fixed on the aluminium stubs and coated with a thin layer of gold. For TEM analysis, the brown clumps were first dispersed in water and ultrasonicated for 10 min. A small drop of suspension was placed on a carbon-coated copper grid and dried under an infrared lamp. Transmission electron micrographs were obtained on JEOL-1011 instrument with an accelerating voltage of 100 kV. The composition of clumps was determined by employing EDX analysis in both the cases.

Plasmid isolation from K. pneumoniae Kpn555

Plasmid DNA was isolated using the standard alkaline lysis method proposed by Sambrook [10]. The isolated product was detected by an agarose gel (1%) run. The product was visualized and compared with a standard 10 kb ladder.

Transformation of the isolated plasmid into Escherichia coli DH5α

Plasmid DNA was isolated from a 50 ml culture in its log phase and was eluted in a TE buffer (10 mM-Tris/HCl/l m-EDTA, pH 8). This isolated plasmid was used to transform 100 μl of E. coli DH5α competent cells by calcium chloride method. These transformed strains were tested for their uptake of plasmid after transformation and increased lead tolerance.

Screening for transformed Escherichia coli DH5α cells and assessment of lead tolerance

The transformed strains were tested for their uptake of plasmid after transformation and increased threshold of lead tolerance. Since K. pneumoniae Kpn555 was isolated fromcoffee pulp waste along with Brevibacterium sp. MTCC10313 [11], it also showed tolerance to caffeine (8 g/L).

The transformed and non transformed colonies of E. coli DH5α were grown in LB medium with different lead concentrations ranging from 100 mg/L to 800 mg/L and their lead tolerance was compared. Bacterial growth was measured by taking OD of bacterial culture at 600 nm.

Extraction and purification of biosurfactants

K. pneumoniae Kpn555 was cultured in NB supplemented with 400 ppm of Pb according to MIC studies performed, (Article, n.d.) for 5 days post which the biosurfactant was extracted from the cell free supernatant according to the protocol. The cell free supernatant was acidified to pH 2.0 with 6MHCl and kept for precipitation overnight at 4°C. The precipitated biosurfactants were separated by centrifugation at 10,000 rpm for 20 min and resuspended in 0.1 M sodium bicarbonate solution. The biosurfactants were purified by extraction thrice with 2:1%v/v chloroform-ethanol mixture at 25°C.

Effect of biosurfactant on heavy metal removal in wastewater

In order to establish if the biosurfactant produced by K. pneumoniae Kpn555 possesses the capability to biosorbPb from its Pb-supplemented culture medium, the purified biosurfactant was analysed for the presence of Pb by atomic absorption spectroscopy (AAS). Pb solutions of known concentrations were employed as the standards.

As a confirmatory test, the purified biosurfactant (100 mg/l) was added to a Pb solution of concentration 200 ppm and was subsequently incubated for 7 days. Then, the residual concentrations of heavy metal were detected using AAS and the bio removal activity was measured using the equation:

%Bioremoval activity=(Ci-Cf)/Ci*100

Where,

Ci is the initial concentration of heavy metal in wastewater before treatment

Cfis the final concentration of heavy metal in wastewater treated with bacterial supernatant

Characterization of purified biosurfactant

Biochemical characterization of biosurfactants:The presence of carbohydrate and protein moieties in the purified biosurfactant was identified by Anthrone and Molisch tests and Folin-Ciocalteu reagents respectively (ABDUL-FADL). The presence of lipid moiety was confirmed by thin layer chromatography using a solvent system of chloroform:methanol:water (70:10:0.5) and subsequent exposure of the plate to iodine vapours.

The produced biosurfactant was quantified by estimating the protein, lipid and carbohydrate concentrations by lowry's method, phosphovanilin method and phenol sulphuric acid method respectively. The standard plots were obtained by employing bovine serum albumin as protein standard, triglyceride as lipid standard and glucose for carbohydrate standard respectively.

Fourier transform infrared spectroscopy

Fourier transform IR (FTIR) spectroscopy is a nondestructive technique for structural characterization of proteins and polypeptides and was performed on the isolated biosurfactant, in the range of 500-4000 cm-1 with an average of 25 scans over the entire range using a Shimadzu FTIR Spectrophotometer. For the analysis, the dry and powdered biosurfactants were dispersed in potassium bromide (Merck, USA) pellets in the ratio, 1:3.

Results and Discussion

Determination of minimum inhibitory concentration (MIC) of lead

The MIC of lead in K. pneumoniaeKpn 555 was found to be 900 mg/L. Beyond this concentration there was complete cessation of the bacterial growth. K. pneumoniae CBL-1 isolated from heavy metal laden industrial wastewater had a MIC of 700 mg/L of lead [12]. However, Marzan et al [13] have reported that Gemella sp. and Micrococcus sp, isolated from tannery effluent could tolerate lead upto 1900 mg/L and

1800 mg/L respectively.

Growth kinetics and biosorption of lead

The concentration of lead added was chosen on the basis of MIC obtained. Growth was found to decrease in the presence of 800 mg/L of lead. Fluctuation was visualised in growth pattern in the stationary phase, which was absent in the control (Figure 1). It could be presumed that, in the presence of lead, probably some genes of K. pneumoniaeKpn 555 get switched on in the stationary phase, leading to the production of some products that help in combating the stress caused by high concentration of lead thereby reducing its toxic effects on the growth of the bacterium.

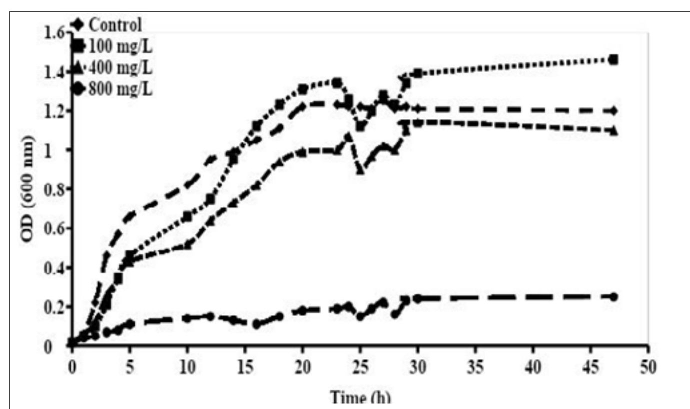

Figure 1: Difference in growth pattern of Klebsiella pneumoniae Kpn555 in the absence and presence of lead.

Also, brown clumps were visualised in the medium only at 100 and 400 mg/L (Figure 2). The clumps attached together to form a mat like structure. The mat was reformed even after the clumps were dispersed suggesting that there could be some binding component which caused the agglomeration.

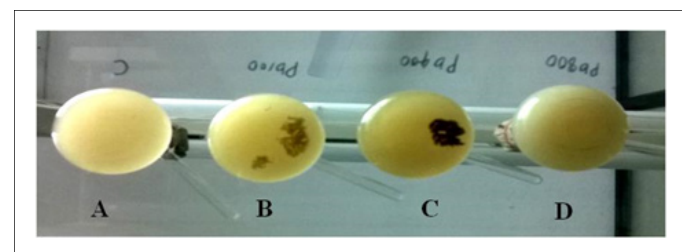

Figure 2: Brown clumps formed on growth of Klebsiella pneumoniae Kpn555 in NB with lead concentration of 100 mg/L (B) and 400 mg/L (C); Clumps absent in NB without lead (A) and in NB with lead concentration of 800 mg/L (D).

On AAS studies it was observed that the maximum biosorption of lead was found in the first 15 h after which it remained constant (Figure 3). This coincided with the irregular fluctuations observed during the bacterial stationary phase. This may probably mean that, as the bacterium reaches the stationary phase, the presence of the lead causes a toxic effect on it due to which it releases some amount of lead back into the medium. Once the bacteria adjust to the lead concentration in the stationary phase, it again continues to biosorb lead on its surface. This can be confirmed from the pattern where in, a slight decrease is observed in allthe three cases at about 45 h. The agglomerates observed in 400 mg/L of lead supplemented medium were lysed and the lysate was subjected to AAS. The cell lysate showed 367 mg/L lead which confirmed that the bacteria indeed formed agglomerates due to the secretion of some binding material and biosorbed lead out of the medium and deposited it on its surface. Aspergillus species isolated from soil samples near the metal plating industry was grown in medium containing different concentrations of lead (0.2-1.5 mM) to determine its resistance to heavy metals and it was observed that the organism was found to accumulate lead particles outside and inside the cell [8].

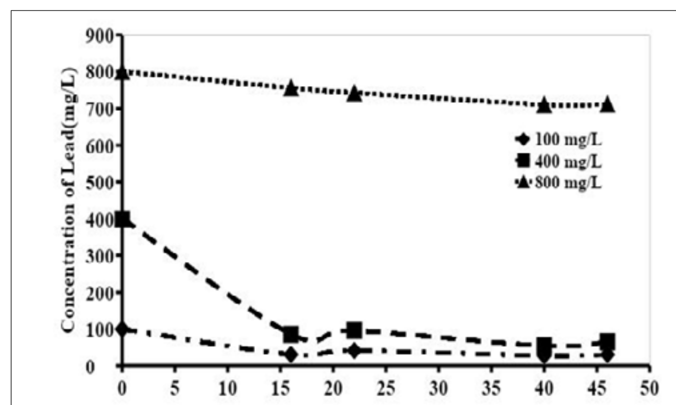

Figure 3: Decrease in concentration of lead from NB supplemented with lead, on growth of Klebsiella pneumoniae Kpn555 for 48 h.

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive spectrometry (EDS) analysis

On subjecting brown agglomerates to SEM analysis, it was seen that the brown agglomerates were clumps of bacteria with some surface deposition. Deposition was also observed in the interstitial spaces (Figure 4A). EDS analysis confirmed the presence of lead in these deposits (Figure 4B). Similar studies conducted by Girisha [2] on Bacillus licheniformis showed a reduction in size of bacterial cells which was attributed to biosorption of lead onto the bacterial cell surface. Enterobacter sp. J1 isolated from wastewater treatment plant also showed surface deposition of lead, copper and cadmium ions on the surface of cells as visualised by SEM [7].

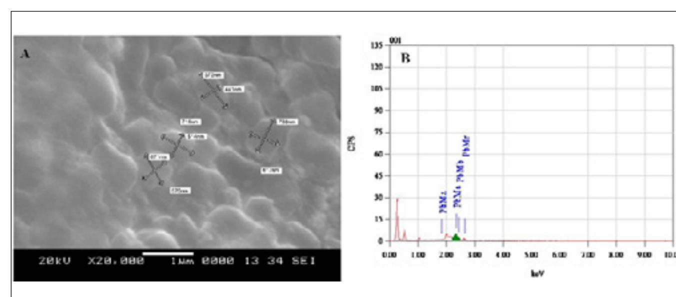

Figure 4: SEM micrograph of brown clumps showing cells of K. pneumoniae Kpn555 with surface deposition (A); EDS analysis confirming presence of lead on the bacterial cell surface (B).

In order to analyse the location of lead in the bacterial cell, the agglomerates obtained were observed by TEM and corresponding EDS analysis. TEM images showed the presence of dark circular deposits in the bacterial cytoplasm which were confirmed by EDS analysis as lead (Figure 5A and 5B). Klebsiella sp. 3S1 isolated from wastewater

treatment plant showed that lead ions were bio accumulated into the cytoplasm and adsorbed on the cell surface [14].

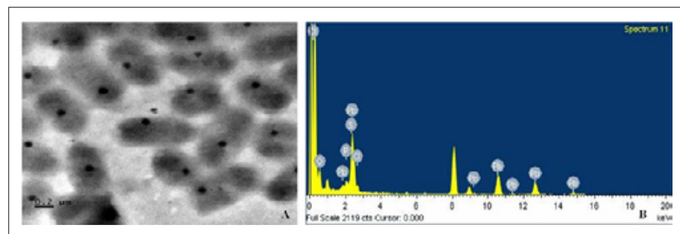

Figure 5: TEM micrograph of brown clumps showing accumulation of lead as circular deposit in the cytoplasm of K. pneumoniae Kpn555 (A); EDS analysis confirming presence of lead in the circular deposits (B).

Plasmid isolation from K. pneumoniae Kpn55 and transformation into E. coli DH5α

The plasmid was isolated from K. pneumoniae Kpn55 using alkaline isolationprocedure and subjected for agarose gel electrophoresis to determine its size. The size of the plasmid could be approximated as 30-40 Kbp (Figure 6). This plasmid was used for transforming E. coli DH5α and the transformed cells showed tolerance upto 6 g/L caffeine whereas the non transformed cells could tolerate only 2 g/L of caffeine (Figure 7).

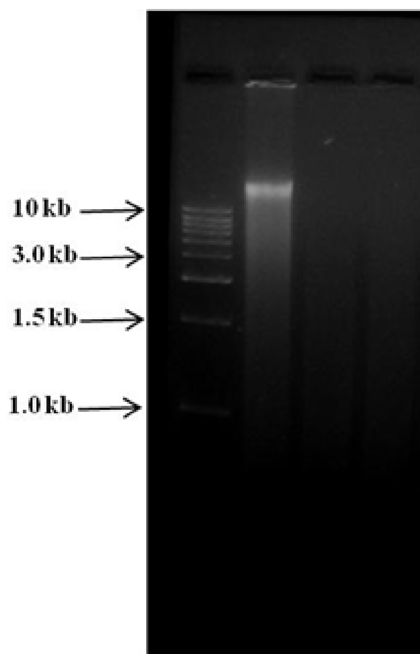

Figure 6: Plasmid isolated from K. pneumoniae Kpn555 (Lane 1:10Kb marker, Lane 2:Plasmid).

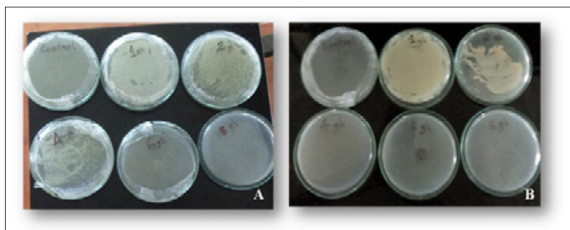

Figure 7: Growth of transformed E coli DH5α colonies till 6 g/L of caffeine (A); Growth of non transformed E coli DH5α colonies till 2 g/L of caffeine (B).

Assessment of lead tolerance

The transformed E. coli DH5α colonies were selected from 6 g/L plate and grown in NB medium supplemented with different concentrations of lead (100, 400 and 800 mg/L). The transformed E. coli DH5α cells grew at all concentrations whereas the non-transformed colonies could tolerate only upto 400 mg/L of lead. The transformed colonies also formed agglomerates in NB supplemented with 400 mg/L lead (Figure 8). This means that, the genetic ability of lead resistance and bioaccumulation of lead by Klebsiellapneumoniae Kpn555 is due to the genes present on the plasmid and not the genomic DNA. Halomonas BVR 1 isolated from an electronic industry effluent could tolerate lead upto 400 mg/L and heavy metal resistance in this strain was also plasmid mediated [15].

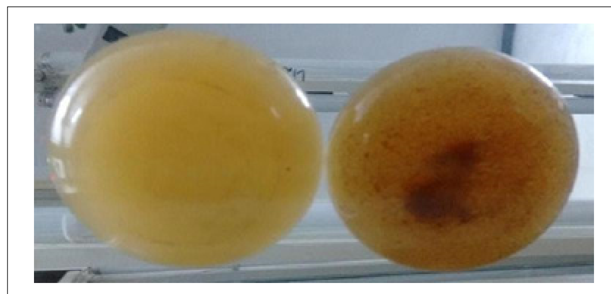

Figure 8: Absence of brown clumps in NB supplemented with 400 mg/L of lead when competent E coli DH5α cells were grown (left); Presence of brown clumps in NB supplemented with 400 mg/L of lead when transformed E coliDH5α cells were grown (right).

The outcome of our study reveals that Klebsiellapneumoniae Kpn555 isolated from coffee pulp waste demonstrates the ability to tolerate biosorp and accumulate high concentration of lead. This capacity of the bacterium was proved to be plasmid mediated by transformation of isolated plasmid into E. coli DH5α. Since Klebsiellapneumoniae is pathogenic and cannot be exploited directly for lead removal from polluted sites, its plasmid could be utilized for the same. Further investigations should be carried out to optimise leadremoval by bacteria transformed with the plasmid from K. pneumoniae Kpn555. These strains could be exploited as a novel bio-sorbent in bioremediation of lead.

Bioremoval of Pb by purified supernatant

AAS results revealed the presence of 8 ppm of lead in the purified extract of lead induced biosurfactant. When the biosurfactant was incubated with Pb solution of concentration 200 ppm, a white precipitate was observed at the end of 1 day. The Pb solution was centrifuged to separate the white precipitate and the clear supernatant was subjected to AAS. The concentration of Pb was found to steadily decrease till the 5th day of incubation after which the Pb concentration plateaued. A Pb concentration of 105 ppm was observed at the end of 5 days (Figure 9). This observation implies that a biosurfactant concentration of 100 mg/L is sufficient for the bioremoval of 50% of total Pb added to the solution. The mechanism of this action can be explained by the plausible interaction between the alkyl halide groups present in the biosurfactant and the Pb ions. A rhamnolipidbiosurfactant isolated from Gram negative Bacilli had the ability to biosorp chromium and it was found to bind to 50% of the chromium concentration present in the sample.

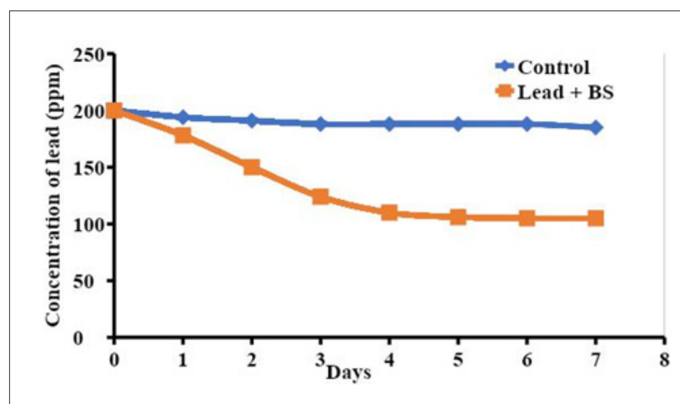

Figure 9: Bioremoval of the lead by the surfactant produced by K. pneumoniae Kpn555.

Characterisation of biosurfactant produced by K. pneumonia Kpn555

The yield of biosurfactant after purification was found to be 1.23 mg/ml. In response to the stress imposed by Pb (400 ppm) on the bacteria, the biosurfactant that was produced by K. pneumoniae Kpn555 was found to exhibit a positive result for Molisch's test while a mild colour change was observed in response to Folin-Ciocalteau test thereby suggesting a lower proportion of protein in the biosurfactant structure. Furthermore, appearance of a yellow spot on the TLC plate in response to Iodine vapours led to the conclusion that the biosurfactant produced is in fact a Glycolipoprotein by nature with a Sugar:Lipid:Protein ratio of 33.76:57.04:9.2.

The FTIR spectra of the biosurfactant produced (Figure 10), exhibited a medium absorption band at 3427.51 cm-1 and 3464.15 cm-1 which implied the presence of O-H groups corresponding to carboxylic acid functional group. The peak observed at 2358.94 cm-1 points to the presence of the C=C functional group. The presence of C=C nitrile group as part of the structure of biosurfactant can also be concluded from the FTIR graph. While the peak observed at wavenumber corresponding to 3076.46 cm-1 was assigned to the C-H group of alkenes/alkenyl functional group, that observed at a wavenumber range of 1015 -1200 cm-1 was assigned to C-O-C/C-O stretches of sugars. A distinct pattern of 3 peaks observed in the wavenumber range of 500-800 cm-1 is suggestive of alkyl halide (C-X) groups, wherein X corresponds to the halogen of alkyl halides. The FTIR results were a reaffirmation of the Glycolipoprotein nature of the biosurfactant produced.

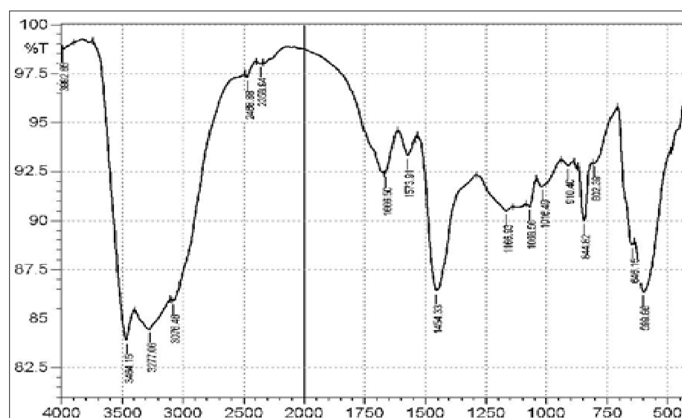

Figure 10: FTIR spectrum of biosurfactant produced by K. pneumoniae Kpn555.

Conclusion

Biosurfactants as a medium of bioremediation of heavy metals is an emerging solution with promising implications. In the current study, Klebsiellapneumoniae Kpn555 was found to be heavy metal tolerant and produce a biosurfactant exhibiting the capability to bioremove 50% of added Pb. The results opened new perspectives for the use of this strain as a promising biosurfactant producer for efficient heavy metal removal. Further research on gene regulation and commercial production of biosurfactant is needed to be robustly employed as an effective and ecofriendly tool towards in situ bioremediation of industrial effluents.

Declaration of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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