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Isolation, Identification and Methanogenesis of CNSL Degrading Bacteria and Immobilized Bioremediation Techniques of CNSL and its Contaminated Water from Cashew Industry

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Abstract: In cashew processing unit, the cashew nut shell liquid is the main contaminating agent. Chemically this liquid contains phenolic compounds like Anacardic acid, Cardol and cardanol. The waste water and effluents from the factory is contaminated with cashew nut shell liquid (CNSL) and cause environmental pollution. The contamination by those compounds is highly risky both for human health as well as for the ecological balance. As the CNSL contains only phenolic compounds, it is liable to polymerization and forms hard uneven surface on the floor of the unit. Again it dislodges working environment of the unit for women workers. The aim was to screen out potential organisms for the bioremediation of CNSL, which contains phenolic compounds such as cardol, methyl-cardol and mainly cardanol. The results showed that five organisms were promising which were screened from a few hundreds of organisms from CNSL contaminated soil. The organisms were tested for their positive nature using water displacement studies which were inoculated onto sterilised CNSL containing cashew shell powder and the methane gas produced was analysed using Gas Chromatography. *Pseudomonas pseudoalcaligenes* strain which was more promising and were further immobilized on baked clay beads and was proved to be effective in treated with waste water and CNSL by reducing the BOD and COD values using an immobilized cell reactor. These beads are active and stable in aqueous media and therefore can be used for the treatment of CNSL at industrial level.

Key words: CNSL · Gas chromatography · *Pseudomonas pseudoalcaligenes* · Baked clay beads · BOD · COD

INTRODUCTION

India ranks third in the production of raw cashew nuts (620,000 tones). The major by-product of cashew nut is the liquid from the pericarp known as cashew nut shell liquid (CNSL). (CNSL) is one of the sources of naturally occurring phenols. CNSL is amber-colored, poisonous, viscous oil obtained from the by-product shells of the cashew nut by extraction. The CNSL content of the cashew (*Anacardium occidentale* L.) raw nut varies between 20 and 25% which contains approximately 70% anacardic acid, 18% cardol and 5% cardanol with the remainder being made up of other phenols and less polar substances. The heat-treated CNSL contains 60–65% cardanol, which is an anacardic acid derivative, having a meta substituent of a C15 unsaturated hydrocarbon chain mainly with 1–3 double bonds [1] .To produce whole cashew nut kernels free of cracks, the operation is done manually, which leaves stains on the floors of cashew processing units and onto the hands of the workers, which are mostly women (Fig.1). Considering the above issue degradation of CNSL is of vital importance.

Today Bioremediation is considered as a new tool to eliminate environment pollutions [2]. Bioremediation**,** which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention as an effective biotechnological approach to clean up a polluted environment. In general, the approaches to bioremediation are environmental modification, such as through nutrient application and

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Fig. 1: Cutting and shelling shed of cashew processing unit contaminated with CNSL spills

Fig. 2: CNSL, cashew shell cake and CNSL contaminated waste water from cashew processing unit

aeration and the addition of appropriate degraders by seeding. Bioremediation offers several advantages over the conventional chemical and physical treatment technologies, especially for diluted and widely spread contaminants [3]. Bioaugmentation is an aspect of bioremediation processes involving addition of natural microorganisms to treat contaminated soil or water. Bioaugmentation, the addition of microorganisms to enhance a specific biological activity, has been practiced intentionally for years in a number of areas, including agriculture and forestry [4] and wastewater treatment [5]. Bak and Widdel [6] studied that only few pure cultures of denitrifying bacteria that is able to use phenol as a sole source of carbon and energy. Zhang *et al*. [7] observed the biodegradation of crude oil by *Pseudomonas aeruginosa* in the rhamnolipids which produced 15.4 gram per litre rhamnolipid cultured in a basal mineral medium using glycerol as a sole carbon source.

In the present study, a new bacterial strain, *Pseudomonas pseudoalcaligenes* has been found to be capable of utilizing CNSL as its sole carbon and energy source. *Pseudomonas pseudoalcaligenes* was isolated from soil contaminated by CNSL and the isolate was immobilized on baked clay beads to examine its degradation ability for CNSL. The objectives of this study were: (1) isolation and identification of potential organisms capable of degrading CNSL (2) to identify the production of methane gas from CNSL using the potential organism (3) immobilization of the most potential strain and to study its effectiveness in degrading CNSL and its contaminated water from cashew industry.

MATERIALS AND METHODS

Materials: CNSL, cashew shell cake and CNSL contaminated waste water (Fig. 2) were collected from the cashew processing units in Kollam. All chemicals used were of analytical reagent grade.

Isolation of Microorganisms and Methanogenesis: Soil samples were collected from five cashew processing industries in Kollam and microorganisms were isolated by mixing each soil sample (10g) with 90 ml sterile distilled water in a 250 ml conical flask to form the soil soup. Each soil soup was serially diluted to get a dilution till 10^{-5}. 1 ml of aliquot from each serial blank was transferred as inoculant onto sterile petridishes with nutrient agar solution and was allowed to solidify. The plates were incubated in a bacteriological incubator at 37°C for 48 hrs. Different bacterial colonies from these plates based on

colony morphology were further streaked on separate nutrient agar slants. Strains showing good performance for degradation of CNSL were further chosen by inoculating a loop of these strains onto 20 ml sterile mineral salts medium in a 100 ml Erlenmeyer flask supplemented with 1% CNSL as the sole source of carbon and energy and incubated in a rotary shaker at 37°C for 120 hrs at 150 r min^{-1}.

The growth containing strains were further tested for their positive nature to degrade CNSL by first inoculating onto 10 ml of sterile nutrient broth in 50ml Erlenmeyer flask and incubated in a rotary shaker at 37°C for 24 hrs at 150 r min^{-1}. This was further inoculated onto 100 ml of sterile CNSL containing 1g of cashew shell powder in a 250 ml Erlenmeyer flask and water displacement study (Fig: 3) was conducted to test its positive nature by producing methane gas. The produced gas was analysed with GC-MS to confirm the methane gas production.

Fig. 3: Water displacement study to test methane production by *E.Coli* strain

Identification of Microorganisms: The isolates named as no.10 and were purified by repeated sub culturing on LB media and these strains was identified by BioMerieux, council for food research and development, konni.

Degradation of CNSL by the Five Different Isolates in Pure Culture: In order to study the biodegradation of CNSL by the five different isolates 5-day BOD test was carried out. Five different bacterial isolate in duplicates were inoculated onto 10 ml sterile nutrient broth in 25 ml Erlenmeyer flask and was incubated at 37°C for 24 hrs at 150 r min^{-1}. 10 ml of CNSL (6 set) in duplicates was diluted to 590 ml with dilution water and was sterilised. 5 set was inoculated in duplicates with 10 ml of the 24 hr old culture. One set in duplicates served as control onto which 10 ml of sterile nutrient broth was added making the dilution of CNSL as 60 times. From each 300 ml sample was taken onto sterile BOD bottles and was incubated in a BOD incubator at 20°C in dark for 5 days. The 5- day BOD test of all the samples including control without microorganism were estimated.

Degradation of CNSL Contaminated Water by Bacteria Immobilised on Baked Clay Beads: Culture No 11 which was more promising by reducing the BOD as estimated were further inoculated onto 250 ml of sterile nutrient broth in duplicates in 500 ml of Erlenmeyer flask and incubated at 37°C for 24 hrs in a rotary shaker at 150 r min^{-1}. Sterile baked clay beads of 5mm size were added onto this culture so the beads are drowned completely. This was further incubated at 37°C in a rotary shaker at 150 r min^{-1} for 24 hrs. To check the viability of the beads 4-5 beads from the stock were added onto sterile 50 ml nutrient broth and this was also incubated at 37°C in a rotary shaker at 150 r min^{-1}. After every 1 hr 0.1ml of this solution was used for spread plate technique and the colonies formed were confirmed based on morphological characteristics. The beads were packed onto a column maintained as batch culture and CNSL contaminated water was fed onto the column and aeration was provided using an aerator for 120 hr. Further the treated CNSL contaminated waste water from cashew industry was dislodged onto a flask and 5-day BOD test and COD (open reflux method) of the treated sample was estimated.

RESULTS AND DISCUSSIONS

Identification of the Bacterial Isolates and Methanogenesis: The isolates numbered 6, 7, 11, 13 and 16 capable of utilizing CNSL as the sole carbon and energy source were isolated from CNSL contaminated soils. The capability of the organisms to degrade CNSL was confirmed based on its ability to produce methane gas which was confirmed by gas chromatography (Fig 4). Cashew shell cake added to the CNSL will serve as extra carbon source thereby methanogenesis is being favoured. All the five potential organisms isolated have been found to be used in different bioremediation practices. The bacterial isolates were identified as *Pseudomonas stutzeri* (No:6), *Enterobacter cloacae* (No:7), *Pseudomonas pseudoalcaligenes* (No:11), *Enterobacter sakazaki* (No:13) and *Sphingomonas paucimobilis* (No:16) based on biochemical analysis. *Pseudomonas pseudoalcaligenes* and *Enterobacter sakazaki* produced methane gas. Sakai *et al*. [8] isolated a methanogen (strain SANAE) belonging to RC-1 from Japanese rice by co-culture with propionate-oxidizing and hydrogen producing *syntrophobacter fumaroxidans*.

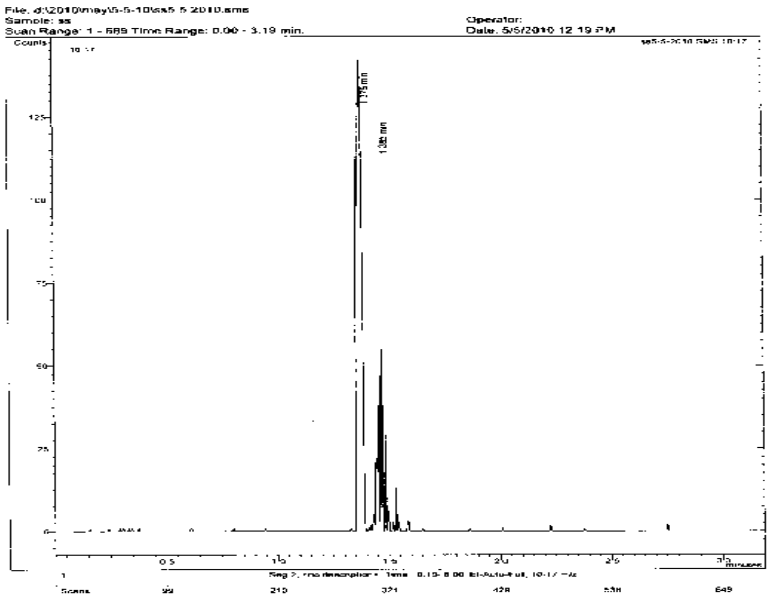

Fig. 4: Production of methane gas was checked with gas chromatography

Table 1: BOD values of CNSL degrading strains

Bacterial culture	BOD (mg/L)
Pseudomonas stutzeri	35
Enterobacter cloacae	48
Pseudomonas pseudoalcaligenes	30
Enterobacter sakazakii	35
Sphingomonas paucimobilis	84

Table 2: BOD and COD values of CNSL contaminated waste water from cashew industry

	BOD(mg/L)	COD(mg/L)
Before Treatment	38.18	2020
After Treatment	11.96	860

Effect of Microorganisms to Degrade CNSL by Reducing the BOD Values: The five different organisms were found to be promising in degrading CNSL by reducing the BOD values (Table 1). From the present study *Pseudomonas pseudoalcaligenes* was found to be more promising in degrading CNSL in comparison with other cultures (Table 1) and this was selected as the target organism for immobilization on baked clay beads. *Pseudomonas pseudoalcaligenes* is gram negative aerobic soil bacterium which has been used in the biodegradation study of phenol. Studies of Rajeswari *et al*. [9] revealed that *Pseudomonas* species degraded the CNSL more efficiently than *Arthrobacter* species and *pseudomonas aeruginosa* MTCC-424.

Annadurai *et al*. [10] reported that biodegradation of phenol using *Pseudomonas pictorum* (23328) a potential biodegrading of phenol was investigated under different operating conditions. Whiteley and colleagues introduce *Pseudomonas pseudoalcaligenes* strain in their research as the main phenol degrading isolate [11,12] and as the CNSL contains mainly phenolic compounds, this study result agrees with previous findings of the ability of the organism to degrade it.

Effect of Bacteria Immobilized on Baked Clay Beads in Degrading CNSL on a Batch Culture by Reducing the BOD and COD Values: *Pseudomonas pseudoalcaligenes* immobilized on sterilized baked clay beads by dipping in 24 hour old culture was found to be active and stable in aqueous media. These beads further packed onto a column with aeration provided was found to be effective in degrading CNSL contaminated waste water from cashew industry by reducing BOD value by 31.32 % and COD value by 33.33% (Table 2). So the treated water can be reused.

CONCLUSION

The data from this study indicate that strain *Pseudomonas pseudoalcaligenes* is a promising candidate for bioremediation project of water and soils contaminated by CNSL than *Pseudomonas stutzeri, Enterobacter cloacae, Enterobacter sakazakii, Sphingomonas paucimobilis*. However, further studies on survival, colonization and population levels of strain *Pseudomonas pseudoalcaligenes* in soil need to be conducted carefully in order to develop more effective

bioremediation. *Pseudomonas pseudoalcaligenes* immobilized on baked clay beads are active and stable in aqueous media and therefore can be used for the treatment of CNSL at industrial level and thus the recycling of CNSL contaminated waste water from cashew industry can be achieved.

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