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Microbiology

Biodegradation of Cashew Nut Shell Liquid by *Delftia acidovorans* and *Pseudomonas aeruginosa* Isolated from Marine Environment

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Abstract

Cashew Nut Shell Liquid or CNSL is a versatile byproduct of the Cashew Industry. CNSL is widely used in paints, varnishes, enamels, laminating resins, rubber compounding resins, cashew cement, polyurethane based polymers and epoxy resins. Biodegradation of paints is a threat to the marine paint industry. CNSL is an eco-friendly antifouling agent widely used in marine paints that prevents fouling of microorganisms to the hull of the ships and boats. Our aim was to isolate, identify and optimize the dominant organism that degrade CNSL and phenol, which would be helpful in developing new paint products that resists these organisms. By enrichment culture technique, a mixed population of microorganisms were obtained. By screening all the microorganisms, it was found that *Delftia acidovorans* and *Pseudomonas aeruginosa* play a significant role in the degradation of CNSL. Degradation of CNSL was estimated spectrophotometrically by Folins-Ciocalteau method at 650 nm. These strains can also be used for bioremediation purposes in CNSL contaminated soils in cashew industries.

Highlights

- Biodegradation of CNSL is possible.
- *Pseudomonas aeruginosa* is the potent degrader of CNSL.

Keywords: CNSL, *Pseudomonas aeruginosa, Delftia acidovorans,* antifouling paint, biodegradation

Cashew nut shell liquid (CNSL), which is a byproduct of cashew industry, is a rich source of unsaturated long chain hydrocarbon phenols (Tyman 1979). Cashew nut shell liquid occurs as reddish brown viscous liquid in the soft honey comb structure of the shell of the cashew nut which is a plantation product obtained from cashew tree, *Anacardium occidentale* L. CNSL is a mixture of phenolic compounds like anacardic acid, cardanol, cardol and 2-methyl cardol. Of these, anacardic acid and cardanol are monohydric phenols and cardol and 2- methyl cardol are dihydric phenols. CNSL has wide applications in polymer-based industries, like paints, varnishes, friction linings, laminating resins, rubber compounding resins, epoxy resins, wood preservatives etc (Kathir and Emerson 2013). Cardanol is extracted from CNSL and is commonly used in the marine antifouling paint that is widely applied to fishing boats, wooden boats, house boats etc. Researches describe the development of a thermoplastic unsaturated polyester resin from cardol isolated from CNSL, and maleic anhydride. (Patrick Muturi Mwangi *et al.* 2013).

Studies shows that *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*. (Sabna Prabha *et al.* 2011). CNSL was found to be degraded more efficiently by *Pseudomonas* species than that *of Arthrobacter* species (Rajeswari *et al.* 2011). *Pseudomonas aeruginosa* was found to degrade phenol at a concentration >80 mg/L and its activity maximum at pH 7 (Bared Razika *et al.* 2010). *Delftia*

acidovorans MC1 was found able to grow on chlorophenoxy herbicides such as 2, 4-dichlorophenoxypropionic acid (2, 4-DCPP) and 2, 4-dichlorophenoxyacetic acid as sources of carbon and energy(Dirk Benndorf *et al.* 2004). Studies on bioremediation potential of *Delftia acidovorans (Comamonas acidovorans)* were also done (Darshan Rudakiya and Kirti Pawar, 2014). Phenol degradation by *Pseudomonas aeruginosa* is maximum in the presence of yeast extract and metal ions like Zinc and Selenium (Shweta and Dhandayudhapani, 2013). Many studies on biodegradation of phenol and phenolic compounds have been reported (Bhavana V Mohite *et al.* 2010; Arunkumar and Anitha 2014; Chakraborty *et al.* 2010; Zia Ur Rahman Awan *et al.* 2013). Phenol degrading ability of azodye degrading strains like *Bacillus cereus* and *Brevibacillus* spp were also checked out by Kumar Sumit *et al.* 2013. Studies on eco-friendly antifouling paints were done (Grant Burgess *et al.* 2003). They highlighted the problems with tin and copper antifouling compounds and the need to develop new environmentally friendly antifouling coatings. In this study we have isolated and identified CNSL degrading bacteria, estimated the extend of degradation of this phenolic compound by Folin-Phenol method (Sadasivam and Manickam, 1996).

Materials and Methods

Sample Collection

Soil and water samples were collected in sterile bottles and bags from different areas of the major fishing Harbor Neendakara and also from Astamudi lake and Thankasseri Port, Kollam, Kerala. The collected samples were brought to laboratory for further studies.

Enrichment and Isolation of CNSL Degrading Bacteria

1g of soil sample and 1 ml of water sample were suspended in 100 ml of mineral salt media containing KH_2PO_4 (0.5 g/L), K_2HPO_4 (1.5g/L), NaCl (0.5 g/L), $MgSO_4.7H_2O$ (0.5 g/L), NH_4NO_3 (1 g/L), $FeSO_4$. 7 H_2O (0.01 g/L), $CaCl_2.H_2O$ (0.01g/L) and NH_4SO_4 (0.5 g/L). 500 mg/L of CNSL was used as a sole source of carbon and incubated at 32°C in 250 mL flask in rotary shaking incubator at 120 rpm for a week. 10ml of the enriched media were transferred asceptically to sterile mineral medium containing CNSL as the carbon source and incubated for a week at 32°C. The enriched medium was then streaked to mineral media agar containing CNSL as the sole source of carbon (Nagamani *et al.* 2009).

Identification of Bacteria

The bacterial cultures obtained as a result of enrichment isolation technique were identified by colony characteristics, Microscopic observation, Gram's staining and biochemical tests. All the biochemical tests were interpreted by VITEK 2 Systems Version:06.01.

Estimation of Catechol Degradation

Bacterial colonies were inoculated into sterile mineral medium broth containing catechol 10 mg/L as sole source of carbon and incubated overnight at 32°C in a shaker at 120 rpm. The 24-hour old culture was centrifuged at 10000 rpm for 10 minutes. The pellet so obtained was added to sterile mineral medium containing 10mg/L catechol and was incubated at 32°C in a shaker at 120 rpm. The decrease in concentration of the phenolic compound was determined by Folins Ciocalteau method at 1hour interval at 650 nm by using UV-VIS–NIR Spectrophotometer (SHIMADZU UV-3600). The percentage degradation of catechol was calculated by the following equation (Shweta and Dhandayuthapani, 2013).

$$\text{Percentage of Degradation} = \frac{(Co - Cf)}{Co} \times 100$$

Optimization of Different Parameters for Biodegradation of Catechol

Effect of p^H on Biodegradation of Catechol

Effect of p^H on the biodegradation of catechol was determined by inoculating the 24-hour bacterial inoculums to sterile Mineral Salt Medium containing 10 mg/L catechol as carbon source at different p^H values (5, 5.5, 6, 6.5 and 7) in 250 ml Erlenmeyer flasks. The cultures were incubated at 32°C in a shaker at 120 rpm. The extent of catechol degradation determined at regular intervals.

Effect of Temperature on Biodegradation of Catechol

Effect of temperature on the biodegradation of catechol was determined by inoculating the 24-hour bacterial inoculums to sterile Mineral Salt Medium containing 10 mg/L catechol as a carbon source in 250 mL Erlenmeyer flasks. The cultures were incubated at different temperatures (28°C, 30°C, 32°C, 34°C and 36°C) in shaker at 120 rpm. The extent of catechol degradation determined at regular intervals.

Effect of Carbon Source on Biodegradation of Catechol

Effect of carbon source on the biodegradation of catechol was determined by inoculating the 24-hour bacterial inoculums to sterile Mineral Salt Medium containing different concentrations of catechol (5mg/L, 10 mg/L, 15 mg/L and 20 mg/L) as a carbon source in 250 mL Erlenmeyer flasks. The cultures were incubated at 32°C in shaker at 120 rpm. The extent of catechol degradation determined at regular intervals.

Effect of Organic Nitrogen Source on Biodegradation of Catechol

Effect of organic Nitrogen source on the biodegradation of catechol was determined by inoculating the 24-hour bacterial inoculums to sterile Mineral Salt Medium containing 10mg/Lcatechol as a carbon source and nitrogen source (peptone, beef extract, yeast extract and tryptone) in 250 mL Erlenmeyer flasks. The cultures were incubated at 32°C in a shaker at 120 rpm. The extent of catechol degradation determined at regular intervals.

Effect of Inorganic Nitrogen Source on Biodegradation of Catechol

Effect of inorganic Nitrogen source on the biodegradation of catechol was determined by inoculating the 24-hour bacterial inoculums to sterile Mineral Salt medium containing 10mg/L catechol as carbon source and nitrogen source ($CaNO_3$, $NaNO_3$,KNO_3 and NH_4NO_3) in 250 mL Erlenmeyer flasks. The cultures were incubated at 32°C in a shaker at 120 rpm.The extent of catechol degradation determined at regular intervals.

Effect of Agitation on Biodegradation of Catechol

Effect of agitation on the biodegradation of catechol was determined by inoculating the 24 hour bacterial inoculums to sterile Mineral Salt Medium containing 10mg/L catechol as carbon source in 250 mL Erlenmeyer flasks. The cultures were incubated at 32°C in a shaker at 120 rpm. The extent of catechol degradation determined at regular intervals.

Effect of Light on Biodegradation of Catechol

Effect of light on the biodegradation of catechol was determined by inoculating the 24-hour bacterial inoculums to sterile Mineral Salt Medium containing 10mg/L catechol as carbon source in 250 mL Erlenmeyer flasks. The cultures were incubated at 32°C in presence and absence of light. The extent of catechol degradation determined at regular intervals.

Estimation of CNSL Degradation

Bacterial colonies were inoculated into sterile mineral media broth containing CNSL 500 mg/L as the sole source of carbon and incubated over night at 32°C in a shaker at 120rpm. The 24-hour old culture was centrifuged at 10000 rpm for 10 minutes. The pellet so obtained was added to sterile mineral medium containing 500mg/L CNSL and incubated at 32°C in a shaker at 120 rpm. The decrease in concentration of the phenolic compound was determined by Folins Ciocalteau method at 1hour interval at 650 nm by using UV-VIS –NIR Spectrophotometer (SHIMADZU-UV-3600).

Growth Determination

To determine the growth, the cells were grown in MSM medium containing catechol ($MSMC_1$) and MSM medium containing CNSL ($MSMC_2$) as the sole source of carbon. After overnight incubation; it was centrifuged at 10000 rpm for 20 minutes. The pellets were added to $MSMC_1$ and $MSMC_2$ medium and kept in shaker at 120 rpm at 32°C. Bacterial growth was obtained by measuring the Optical density at 595nm (Jyothi *et al.*2012).

Estimation of Biomass

Biomass concentration was estimated by Dry cell weight method. After overnight incubation of the bacterial cultures in $MSMC_1$ and $MSMC_2$ medium, centrifuged at 10000 rpm for 20 minutes. The pellet so obtained was added to $MSMC_1$ and $MSMC_2$ medium. At regular intervals, the medium was filtered through pre-weighed dry Whatmann no.1 filter paper. The filter paper was dried in oven at 70°C overnight and cooled and again weighed. The difference between pre-weighed filter paper and final weight gives the biomass concentration.

Results and Discussion

Isolation and Identification of CNSL Degrading Bacteria

A total of 22 CNSL degrading bacteria were isolated. The isolates were screened and two bacterial isolates were selected for our study. Both the bacteria were found to be Gram-negative and analysis confirmed it as *Pseudomonas aeruginosa* and *Delftia acidovorans.* The biochemical details are shown in Table 1.

Table 1. Biochemical Details of isolated organisms

	Pseudomonas aeruginosa	*Delftia acidovorans*
APPA	–	–
ADO	–	–
PyrA	–	+
IARL	–	–
dCEL	–	–
BGAL	–	–
H_2S	–	–
BNAG	–	–
AGLTp	+	–
dGLU	+	–
GGT	+	+
OFF	–	–
BGLU	–	–
dMAL	–	–
dMAN	–	–
dMNE	+	–
BXYL	–	–
BAlap	+	–
ProA	+	+
LIP	–	–
PLE	–	–
TyrA	+	+
URE	+	–
dSOR	–	–
SAC	–	–
dTAG	–	–
dTRE	–	–
CIT	+	–
MNT	+	–
5KG	–	–
ILATk	+	+
AGLU	–	–
SUCT	+	+
NAGA	–	–
AGAL	–	–
PHOS	–	–
GlyA	–	–
ODC	–	–
LDC	–	–
IHISa	–	–
CMT	+	+
BGUR	–	–
O129R	+	–
GGAA	–	–
IMLTa	+	–
ELLM	–	+
ILATa	+	–

Estimation of Catechol Degradation

Estimation of Catechol degradation by *Pseudomonas aeruginosa* and *Delftia acidovorans* showed that after four hours of incubation *Pseudomonas aeruginosa* and *Delftia acidovorans* utilized about 99.15% and 67.35% of Catechol respectively. This was supported by HankD. *et al.* 2010, Hence among the dominant catechol degraders, *Pseudomonas aeruginosa* was found to be most efficient. Results were shown in Figure 2.

Optimization of Different Parameters for Degradation of Catechol

To determine the optimum conditions for maximum degradation of phenolic compounds by *Delftia acidovorans* and *Pseudomonas aeruginosa*, catechol was used as the carbon source and various parameters such as effect of p^H, temperature, carbon source, organic nitrogen source, inorganic nitrogen source, agitation and presence of light were done and are shown in Figures 3-9. Both *Pseudomonas aeruginosa* and *Delftia acidovorans* showed maximum activity at neutral p^H, 32 ^{o}C-36 ^{o}C temperature. This was supported by Chakraborty *et al.* 2010 and Polymenakou and Stephanou, 2005. Low concentration of carbon source showed maximum activity which supports Ahmed *et al.* 1995. It was found that in the presence of inorganic nitrogen source, ammonium nitrate, both bacteria showed maximum activity than other inorganic and organic nitrogen sources. Degradation was observed to be maximum in presence of light and when agitated at 120rpm.

Estimation of CNSL Degradation

The above optimized conditions were provided, and CNSL was used as the carbon source, both *Pseudomonas aeruginosa* and *Delftia acidovorans* degraded CNSL to about 85.36% and 79.43% respectively after 4 hours of incubation (Figure 10). This supports the work by Rajeshwari *et al*. 2011. They found that *Pseudomonas aeruginosa* degrades CNSL faster than other microbes.

Growth Determination

Bacterial growth was obtained by measuring the Optical density in $MSMC_1$ medium and $MSMC_2$ medium containing the bacterial inoculums at 595nm and is shown in Table 2 and 3. It shows that maximum degradation of catechol occurs within 3 hours after inoculation, and that of CNSL occurs within 6 hours after inoculation of both bacteria.

Fig. 1. Bacterial colonies in MSM containing CNSL as sole source of carbon

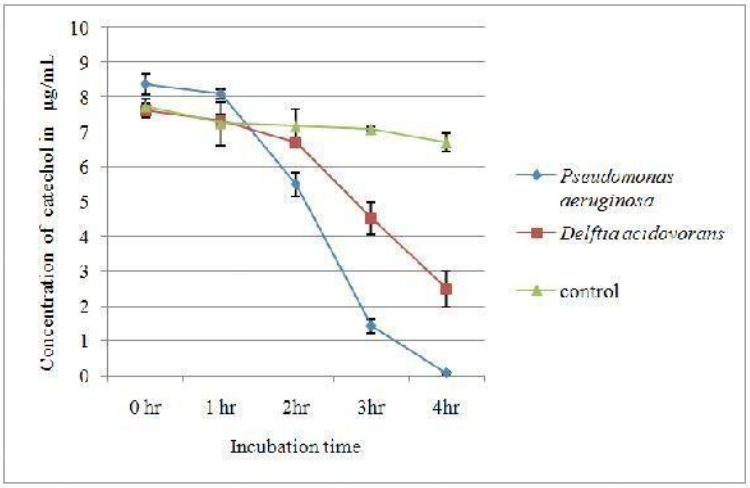

Fig. 2. Catechol degradation by *Pseudomonas aeruginosa* and *Delftia acidovorans*. Values expressed as means ±SD (n=3)

Optimization of Different Parameters for Degradation of Catechol

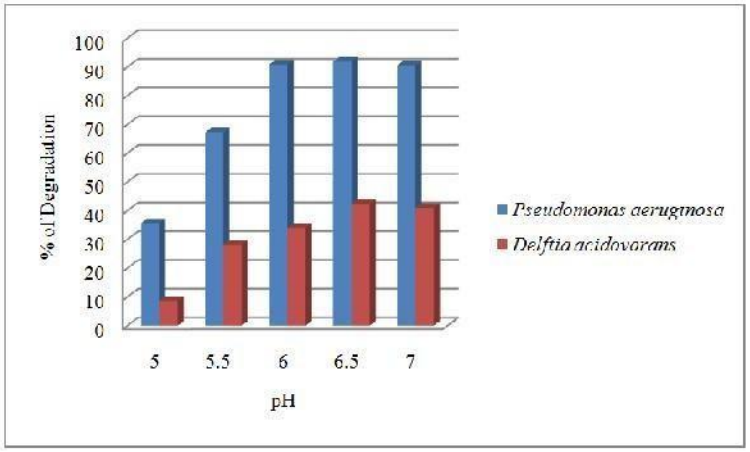

Fig. 3. Effect of different p^H on degradation of catechol by *Delftia acidovorans* and *Pseudomonas aeruginosa*

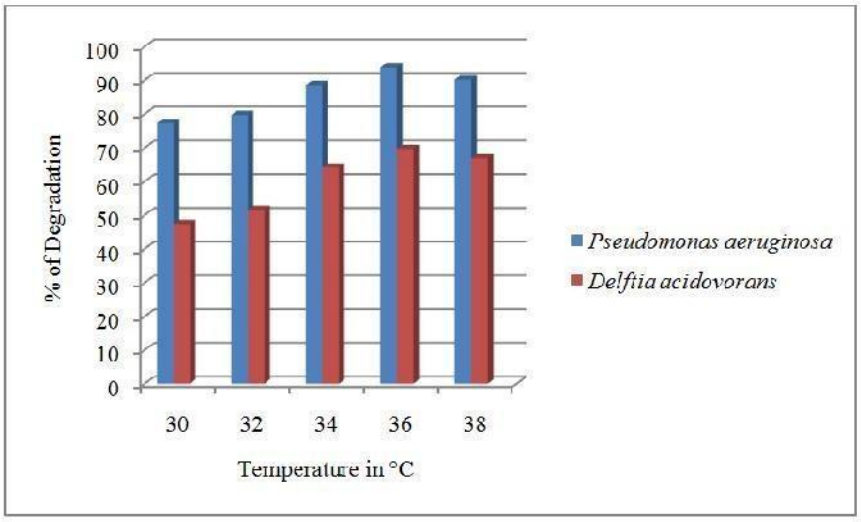

Fig. 4. Effect of different temperatures on degradation of catechol by *Delftia acidovorans* and *Pseudomonas aeruginosa*

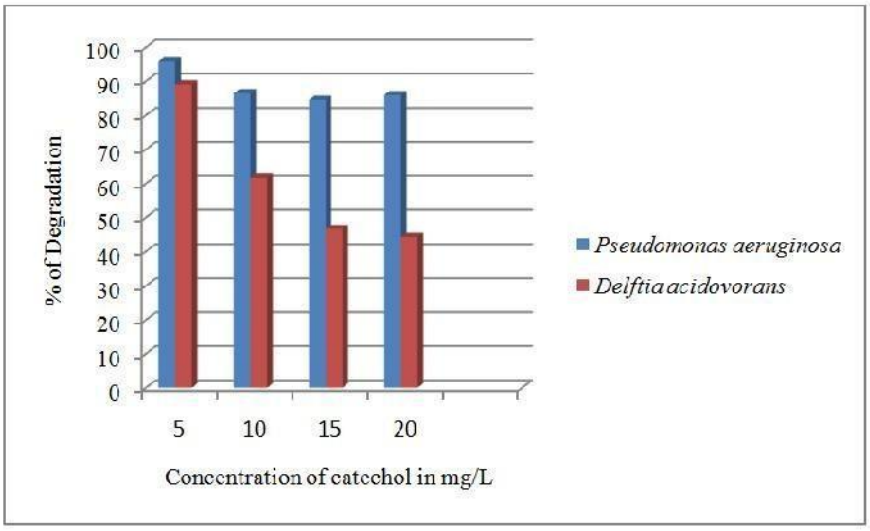

Fig. 5. Effect of different concentrations of carbon source (catechol) on degradation by *Delftia acidovorans* and *Pseudomonas aeruginosa*

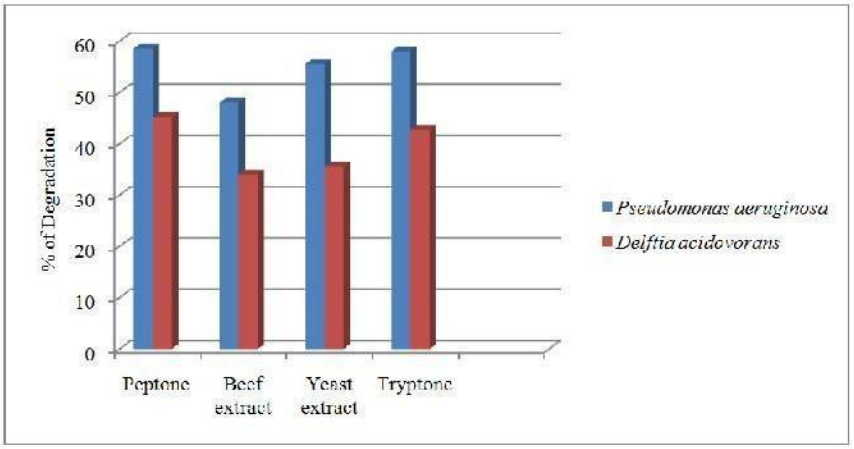

Fig. 6. Effect of different organic nitrogen source on degradation of catechol by *Delftia acidovorans* and *Pseudomonas aeruginosa*

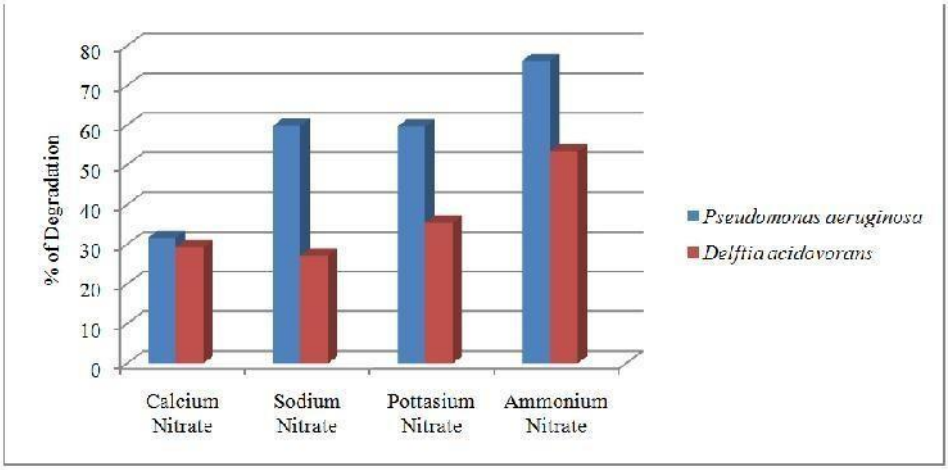

Fig. 7. Effect of different Inorganic nitrogen source on degradation of catechol by *Delftia acidovorans* and *Pseudomonas aeruginosa*

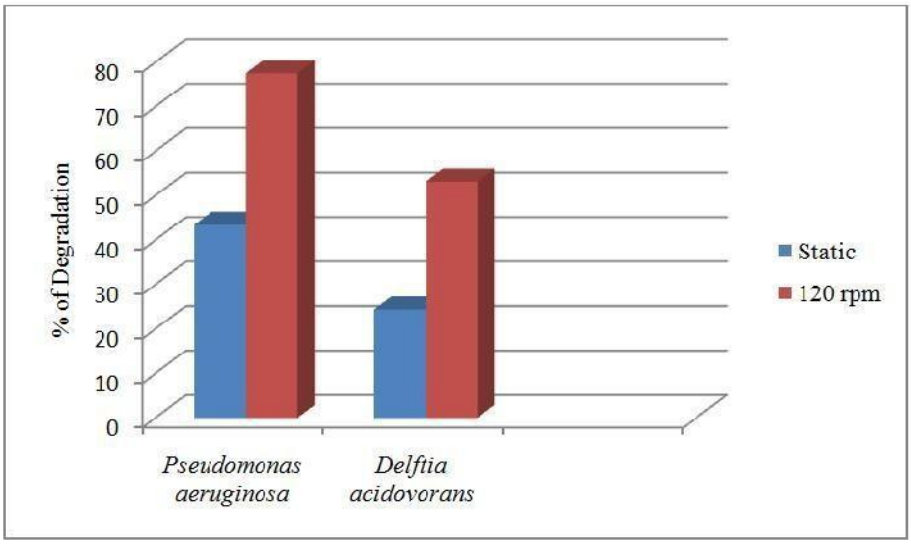

Fig. 8. Effect of agitation on degradation of catechol by *Delftia acidovorans* and *Pseudomonas aeruginosa*

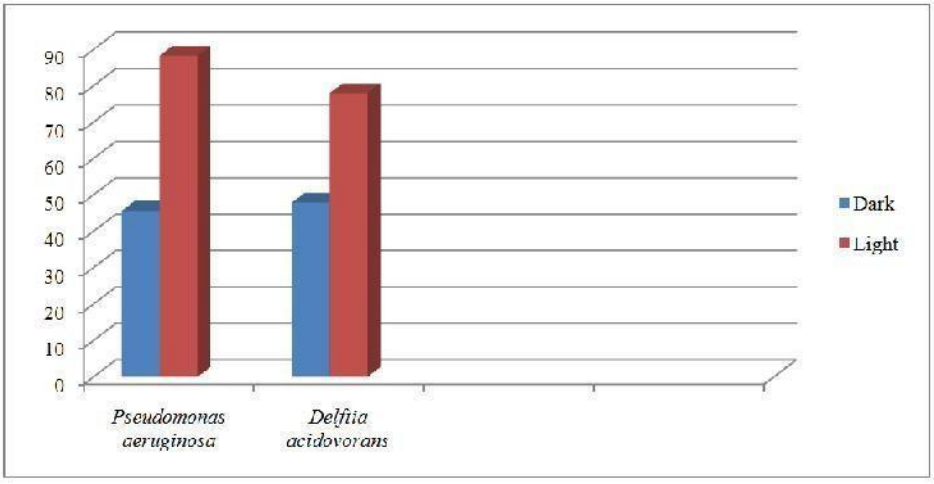

Fig. 9. Effect of presence of light on degradation of catechol by *Delftia acidovorans* and *Pseudomonas aeruginosa*

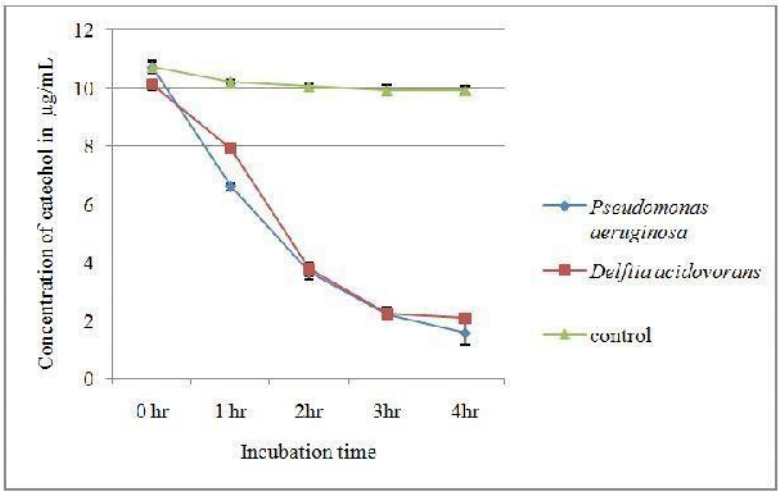

Fig. 10. Degradation of CNSL by *Pseudomonas aeruginosa* and *Delftia acidovorans.* Values expressed as means ± SD (n=3)

Table 2. Optical density of bacterial growth in $MSMC_1$ medium at 595 nm.

Bacteria	Af ter 1 hr	Af ter 2 hr	Af ter 3 hr	Af ter 4 hr
Pseudomonas aeruginosa	0.025 ± 0.003	0.033 ± 0.003	0.035± 0.002	0.025 ± 0.001
Delftia acidovorans	0.02 ± ±0.001	0.026 ± 0.002	0.023 ± 0.001	0.021 ± 0.001

Values expressed as means ± SD (n=3).

Table 3. Optical density of bacterial growth in $MSMC_2$ medium at 595 nm

Bacteria	Af ter 2 hr	Af ter 4 hr	Af ter 6 hr	Af ter 8 hr
Pseudomonas aeruginosa	0.515 ± 0.006	0.616 ± 0.005	0.75 ± 0.03	0.54 ± 0.02
Delftia acidovorans	0.535 ± 0.013	0.64 ± 0.03	0.76 ± 0.01	0.62 ± 0.04

Values expressed as means ± SD (n=3).

Estimation of Biomass

Biomass concentration was estimated by Dry cell weight method (Table 4, 5). The maximum degradation of catechol in $MSMC_1$ was obtained after 3 hours of inoculation and the dry cell weight of *Pseudomonas aeruginosa* was found to be 47.33 ± 3.05 mg/10 mL of the medium. And that of *Delftia acidovorans* was found to be 25.66 ± 5.85 mg/10 mL of the medium and after 6 hours in $MSMC_2$ medium, it was found to be 31.00 ± 1.00 and 34.33 ± 2.30 mg/10 mL of the medium.

Table 4. Estimation of biomass in mg/10 mL of $MSMC_1$ medium

Bacteria	Af ter 1 hr	Af ter 2 hr	Af ter 3 hr	Af ter 4 hr
Pseudomonas aeruginosa	19.33 ± 4.04	36.33 ± 1.52	47.33 ± 3.05	16.00 ± 4.00
Delftia acidovorans	15.00 ± 3.60	19.00 ± 1.00	25.66 ± 5.85	19.00 ± 1.00

Values expressed as means ± SD (n=3).

Table 5. Estimation of biomass in mg/10 mL of $MSMC_2$ medium.

Bacteria	Af ter 2 hr	Af ter 4 hr	Af ter 6 hr	Af ter 8 hr
Pseudomonas aeruginosa	31.66 ± 0.57	32.33 ± 0.57	31.00 ± 1.00	30.33 ± 0.57
Delftia acidovorans	27.33 ± 0.58	28.66 ± 0.57	34.33 ± 2.30	33.33 ± 4.90

Values expressed as means ±SD (n=3).

Conclusion

From the study, we can conclude that *Pseudomonas aeruginosa* and *Delftia acidovorans* are potent phenolic compound degraders, especially CNSL. As the CNSL is widely applied in marine paints, it would be helpful in developing new paint products that resists these organisms. Also, CNSL contamination in soil is the major problem faced by cashew industries. These strains can also be used for bioremediation purposes in CNSL contaminated soils in cashew industries.

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