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Optimization of various Physiochemical Parameters to Enhance 2- Naphthalene Sulfonate Biodegradation by an indigenous Bacterial isolate C2

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ABSTRACT: 2-Naphthalene sulfonate (2-NS), a textile dye intermediate, is highly toxic and has limited biodegradability which poses a threat to aquatic life and human health. Enrichment of sludge from treatment plant receiving textile effluent resulted in isolation of 2-NS degrading bacteria, designated as Isolate C2. To achieve maximum biodegradation activity, optimization of different factors such as growth media, 2-NS concentrations, pH, incubation temperature, inoculum level, and time interval was performed using one factor at a time approach. The best degradation ability (100%) was obtained in 8h, when cells were grown in Sulfur free Mineral Salt Medium using OD_{540} 1.0, pH 7 at 30°C. Isolate C2 was versatile enough to show growth and degradation at 2-NS concentrations ranging from 25-200 mg/L. In nutshell, the study revealed that 2-NS was efficiently consumed by C2 as the sole carbon source and therefore is as an important tool in the bioremediation of wastewater effluent.

Keywords: Biodegradation, Optimization, 2- Naphthalene sulfonate, textile effluent, textile dye intermediate, degrading bacteria, physiochemical parameters.

INTRODUCTION

It is a well known fact that the industrial sector contributes significantly to the growth and economy of developing as well as developed countries around the world, employing millions of people worldwide (Moyo *et al*., 2022). Textile industry is one of the emerging and globally spreading contributors, generating revenue of around 1 trillion dollars and contributing 7% to the total export (Lellis *et al*., 2019). Despite indubitable importance, this industry also carries a negative reputation of being the major environment polluter, having worrisome environmental impact. It has been estimated that textile and dyeing industry contributes to approximately 17–20% of water pollution (Ajmal *et al.*, 2014). Different wet and dry production processes consume large proportion of clean water, non-biodegradable toxic dyes and dye intermediates. Therefore, the wastewater generated from these factories is highly colored, has high BOD, COD, TSS and contains diverse types of persistent pollutants (Al-Tohamy *et al*., 2022).

Aromatic sulfonates is one such major group of organic compounds commonly used as dye assisting chemicals and are found in large proportion in these effluent waters. Naphthalene sulfonates and its analogs, especially 2-Naphthalene sulfonate, is an important representative of this class, commonly identified in textile and tannery wastewaters (Song *et al*., 2005). They have no or limited biodegradability and high water-solubility due to their highly sulfonated nature and aromatic ring. Due to this, these organic compounds can persist in the wastewater unaffected for longer time duration and disposal of this untreated textile effluent into the natural streams and water bodies leads to undesirable effect on the ecosystem and has a detrimental effect on aquatic life, microorganisms as well as human health. Thus, it's absolutely necessary to treat the effluent water before discharging it into waterbodies. Various conventional physico-chemical treatment methods are available, which aim to reduce the pollution parameters in the wastewater; however they have certain limitations like cost-extensiveness, production of toxic sludge posing disposal issues and ability of certain pollutants to resist breakdown by these methods (Suresh, 2014). Therefore, alternative approaches, utilizing microbiological systems, are now being explored more exclusively (Moyo *et al*., 2022), as there are reports on degradation of textile dyes and intermediates by microbial strains (Song *et al*., 2005; Kochher and Kumar 2011; Deshmukh *et al*., 2013; Jackson and Gomathi 2021). It has been reported that the site contaminated with a said pollutant has higher chances of obtaining its potential degraders also, due to their better adaptability and induction of specific metabolic pathways (Bala *et al*., 2022; Azubuikee *et al*., 2016).

In light of this, the present study aimed to isolate the microbial diversity capable of degrading 2-Naphthalene sufonate (2-NS) from sewage treatment plant receiving textile effluent from various textile and dye stuff industries and study the best physiological conditions which would support the maximum efficiency of the most potent bacterial degrader.

MATERIAL AND METHODS

Chemicals and Growth Medium. 2-Naphthalenesulfonate (2-NS) and other required solvents were obtained from Hi Media Laboratories Pvt. Ltd., Maharashtra. Culture Media used in the study, *i.e.*, Minimal Salt Medium (MSM) and Sulfur-free MSM were prepared as given in Khehra *et al*. (2005).

Sample Collection. The sludge sample was collected from Treatment Plant, Ludhiana, Punjab; which receives effluent from various textile industries**.** Upon collection, the samples were immediately stored in refrigeration until further experimentation.

Isolation and Screening of 2-NS degrading bacterial isolates. The isolation and screening was undertaken using protocols described in Kumar *et al*. (2020), where the microbial diversity in the sludge sample was enriched in the presence of 20mg/L to100 mg/L of 2-NS. The isolates, thus obtained, were maintained on N/10 Luria Bertani agar plates supplemented with 2-NS (100 mg/L) and screened for their ability to degrade 2-NS. The isolated cells were grown in MSM having 20mg/L concentration of 2-NS at 30°C for 24h under shaking condition. Following which the absorbance of the supernatant was noted at 200-400 nm by UV–Visible spectrophotometer (UV-1601, Shimadzu, Japan).

Optimization of various physiochemical parameters for obtaining best degradation potential of isolate C2. The effect of various physiochemical parameters on the metabolic and degradation potential of the isolate C2 was evaluated using One Factor at a time (OFAT) approach. For these studies, the preparation of activated cell suspension as well as the measurement of degradation potential of the isolate was done as per protocol outlined in Kumar *et al*. (2020), unless specified otherwise. The effect of Different Growth Media [Minimal Salt Medium (MSM) and Sulfur- free Mineral Salt Medium (SFMSM)]; inoculum level (in terms of initial OD_{540}, *i.e.*, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5); different concentrations of 2-NS (25 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L); initial pH of the growth medium (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0) and incubation temperature (20°C, 25°C, 30°C, 35°C, 40°C and 45°C); Different Time interval (Control, 2h, 4h, 6h, 8h) on degradation potential of isolate C2 was evaluated.

Analytical studies of metabolites formed after biological transformation. High Performance Liquid Chromatography (HPLC) analysis was used to analyze the biological transformation of 2-NS by isolate C2.

Liquid- Liquid Extraction. The samples (abiotic control and biologically treated) were twice subjected to liquid-liquid extraction using ethyl acetate and the pooled organic portion was concentrated using rotary vacuum evaporator (Buchii, Switzerland), filtered through 0.22µ membrane filter (Pall Corporation, USA) and used for further analysis.

High Performance Liquid Chromatography (HPLC) analysis. It was carried out using HPLC (Dionex, USA) containing Restek C-18 column (250mm×4.6mm, 5µm particle size, Restek, USA), having an oven temperature of 30°C and a PDA detector (UVD340U, Dionex, USA), working at 225 nm. The 20 µL sample was injected at the rate of 0.2 mL/min and eluted using the suitable mobile phase.

Statistical analysis. The values expressed in the graphs are represented as mean± SEM (Standard error of means).

All the experiments were conducted at Department of Microbiology, Guru Nanak Dev University, Amritsar; during the academic year 2021-22.

RESULTS AND DISCUSSION

Optimization studies for isolate C2. The ability of isolate *Comamonas* sp. C2 to degrade 2-NS compound was also evaluated under different physiological conditions such as different growth medium, 2-NS concentration, inoculum level, initial pH of the growth medium, incubation temperature, Time interval etc.

Effect of different growth medium. The degradation efficacy of the isolate C2 (at OD_{540} of 0.1) was seen at different time intervals (4 h- 24 h) in Sulfur-Free MSM (SF MSM) and MSM, supplemented with 2-NS (100 mg/L) (Fig. 1). It was observed that after 8h of incubation, 59% and 24% of 2-NS was degraded by the isolate, when grown in SF MSM and MSM, respectively. This efficiency significantly increased to 78% (in case of SF MSM) after 12 h, while it was low (47%) for MSM. Further, 2-NS was completely degraded (100%) by the isolate grown in SFMSM in 16h, while cells grown in MSM took 20h for 100% degradation of 2-NS. Therefore, due to higher degradation efficiency and better growth of the isolate in SFMSM medium, it was selected as a growth medium for further experimentation. It may be because of the ability of the isolate to more efficiently remove sulphite group from 2-NS and utilize it as a source of energy.

Effect of different inoculum levels. The effect of increasing the inoculum levels of the activated cells of isolate C2 to SFMSM, from OD_{540} equivalent to 0.1 to 1.5, on degradation of 2-NS (100 mg/L), was evaluated. It was observed that complete degradation of 2-NS (100%), in medium inoculated with initial inoculum of OD_{540} equivalent to 0.1, was achieved in 16 h; whereas, it took 14 h for the isolate to completely degrade 2-NS, when the initial OD_{540} of cells was set at both 0.25 and 0.5, respectively (Fig. 2). Further increase in OD_{540} to 0.75 and then 1.0 resulted in a significant rise in degradative activity as the complete degradation of 2-NS was done in 12 h and 8 h, respectively. However, further increase in initial inoculum level (OD_{540} equivalent) to 1.25 and 1.5 did not show any increase in the degradation efficiency, when compared to OD_{540} equivalent to 1.0. In light of these observations, further studies were carried out at with an initial inoculum level of OD_{540}1.0. Our finding is in consonance with a study, where degradation of 2-Hydroxyquinoxaline (2-HQ), an aromatic and bicyclic compound obtained from agricultural soil, by *Bacillus* sp. was best carried out at an inoculum density of 1.0 OD (Reddy *et al*., 2014).

Effect of Different 2-NS concentrations. The isolate C2 (at OD_{540} of1.0) was also studied for its degradation efficiency in the presence of different concentrations of 2-NS (25mg/L-200mg/L) in SFMSM medium, at different time intervals ranging from 2h to 48h (Fig. 3). It was observed that the cells growing in the presence of 25mg/L and 50mg/L 2-NS were able to completely degrade it after 4h and 6h of incubation, respectively**.** The higher concentration of 100 mg/L 2-NS was completely degraded after 8h, where 81% of this concentration was degraded by the isolate in first 6h of incubation itself. Upon further increasing the concentration of 2-NS to 150mg/L and 200mg/L, the degradation efficiency of the isolate was negatively affected and slowed down, being achieved after 24h of incubation. Similarly, exposure of isolate to 200mg/L concentration resulted only in its 41% degradation after 8h, while complete degradation was achieved at 48h of incubation. Based on these observations, further studies were carried out using 100 mg/L of 2-NS supplemented to SFMSM.

The effect was also seen on the cell growth, which after 8h, started to slow down or reduced upon extended incubation time. This may be attributed to certain level of direct toxicity of such high concentration of 2-NS on the actively metabolizing inoculant cells or repression of the catabolic pathway's expression. However, it is worth noting that the isolate was able to completely degrade such a higher concentration of the compound, despite taking a comparatively longer time. This highlights the efficiency and versatility of the isolate. This study is in consonance with a report, where *Comamonas* sp. 4BC was able to degrade 100mg/L and 500 mg/L of Naphthalene-2-sulphonic acid (2-NS) within 33h (Song *et al*., 2005). Our finding are in line with another study on a bacterial consortium, capable of degrading the linear alkylbenzene sulfonate (LAS), was isolated from the wastewater treatment plant. It degraded only 68% of 200ppm concentration in 96h; where our study showed better results, as 200ppm 2-NS was completely degraded in 48h. The degradation ability of this consortium was considerably reduced as concentration of LAS was increased beyond 200ppm upto 700ppm; which was attributed to an increase in membrane permeability that causes the dissipation of ion gradients and membrane potential or leakage of essential cell constituents. This could be the possible reason also in reduced cell growth and longer degradation time taken by our isolate C2 (Khleifat, 2006). Our study is better than another report, where only 50 mg/l of an organophosphate, Diazinon in MSM was degraded by *Serratia marcescens* DI101; but within a longer time span of 11 days (Abo-Amer, 2011).

Effect of different time intervals. The degradation efficiency of isolate C2 was also noted at different time intervals of incubation, after addition of the isolate to SFMSM containing 2-NS (100 mg/L), at inoculum level (OD_{540}) of 1.0 (obtained above). It was observed that at a time interval of 2 h, only 18% degradation was achieved (Fig. 4). After 4h, 46% of 2-NS was found to be significantly degraded by the isolate and after 6 h, this degradation rate further increased to 78%. However, complete (100%) degradation of 2-NS (100 mg/L) was achieved by the isolate at 8h.

Effect of initial pH of the growth medium on 2-NS degradation. The effect of pH of the growth medium on the degradation efficiency of isolate C2 was also evaluated after different time intervals of 8h, 24h and 48h (Fig. 5). It was observed that at 8h time interval, the isolate was unable to degrade 2-NS at a medium pH of 4.0 (and remained undegraded even after 24h and 48 h). The medium pH 5.0 supported very low degradation efficiency of the isolate, as only 16% of compound was degraded after 8h and this % remained unchanged even after 24h & 48h. However, when the pH of the medium was set between 6.0 to 10.0; significant increase in degradation efficiency of the isolate was observed even at 8h incubation; where the compound was 86%, 100%, 92%, 88% and 81% degraded at the respective pH (6, 7,8, 9, 10) and reached complete (100%) degradation after 24h. The most effective degradation occurred at pH 7.0 in 8h only. With further increase in pH to 11, the degradation potential was lowered significantly to 61% at 8 h itself and was unaffected after 24h and 48h also. It was worth noting that at pH 12.0, the degradation efficiency of the isolate was completely lost as no degradation of 2-NS was observed at any of the three tested time intervals.

The results obtained in this study are of importance because pH of the growth medium also significantly affects the degradation potential of microbes .pH is an essential environmental parameter that affects activities like cell membrane transport and catalytic reaction balance., or the production of certain metabolites. The structure/function of biological macromolecules, especially proteins, depends on the pH. Therefore, it should be taken into consideration for increased efficiency of the bioremediation system (Nortemann *et al*., 1986). It was worth noting that the isolate C2 was able to sustain growth and its degradation ability at both neutral and alkaline pH (pH 6-11), which proves the versatility of the isolate to tolerate significant variation in pH, which is supported by Patel et al. (2012), who reported 100% naphthalene degradation at alkaline pH 8.0–10.0. The isolate C2, however, showed maximum degradation efficacy at pH 7. This observation is supported by the fact that the intracellular pH of bacteria is close to neutrality and remains almost constant in order to preserve the metabolic capacity and cellular integrity (Sanchez-Clemente *et al*., 2018). Our finding corroborates well with Wang *et al*. (2019), which states that favourable pH range 6.0–8.0, for the growth of microorganisms for biodegradation Our finding (optimal pH 7.0) is in line with previous studies, where, pentachlorophenol degradation by *Pseudomonas* sp. was done rapidly at pH values from 6.3 to 8, where maximum rate of PCP degradation was at pH 6.3 (Wolski *et al*., 2006). Similarly, Diazinon (an organophosphate) was efficiently degraded by *Serratia marcescens* DI101 at pHs from 7.0 to 8.0 (Abo-Amer, 2011). Our finding is in agreement with studies, where 4-chloroaniline (4-CA). (300 mg/l) was completely degraded by *Thauera* sp. M9, when grown in medium

with an initial pH of 7.0 (Kumar *et al*., 2020).While in another study, *Pseudomonas* sp. EN-4 was able to metabolize 4-bromophenol (4-BP), with a maximum transformation of 99.22 % achieved at pH 7.0 (Mahajan *et al*., 2022).

Effect of incubation Temperature. The effect of different incubation temperature (ranging from 20°C to 45°C) on the degradation of 2-NS (100mg/L) by the isolate C2 (OD_{540} of 1.0) was also evaluated after 8h, 24h and 48h (Fig. 6) It was observed that the cells, after 8h of incubation at 20°C and 25°C, did significant degradation of 2-NS, *viz*., 71% and 89%, respectively. The compound was, however, completely degraded (100%) after 24h of incubation. Upon increasing the incubation temperature to 30°C, the cells achieved 100% degradation of 2-NS after 8h incubation. However, when the cells were incubated at 35°C and 40°C, low degradation efficiency was exhibited by the cells (81% and 42%, respectively) after 8h incubation, which somehow increased to 100% and 91%, respectively, after 24h. At a higher temperature of 45°C, the cells could not degrade the 2-NS compound after 8h incubation, but were able to degrade 38% of it after 24 h. The degradation efficiency achieved by the cells of isolate C2, at different incubation temperatures, after 24h remained unchanged even after 48 h of incubation. Since complete degradation of 2-NS was achieved by the cells in just 8h, when incubated at a temperature of 30°C, therefore, this incubation temperature was used for isolate C2 in further experimentation. The results obtained here hold significant importance and are of great value because temperature affects the microbial growth and metabolism. If temperatures gets too high, enzyme activity will diminish and the protein (the enzyme) will denature. On the other hand, lowering temperature will decrease enzyme activity, while at freezing temperatures enzyme activity can stop. It was observed that with increasing the incubation temperature from 20 to 30°C, the degradation efficiency of the isolate increased from 71% to 100% degradation of 2-NS after 8h incubation, but incubation at 35°C and 45°C lowered the degradation efficiency. This lowering of degradation potential at higher temperature may be due to thermolabile nature of proteins/enzymes, which also affects the growth and metabolic functions of microorganisms **(**Tas and Pavlostathis 2014**).** The effect of temperature in our study goes well in line with another study, where the most rapid degradation of diazinon by strain DI101 was recorded at 20°C, 25°C, and 30°C**,** while the slowest degradation was determined at the two extreme temperatures (10 and 40°C), respectively, as seen in our study where 20°C, 40°C and 45°C showed slowest degradation (Abo-Amer, 2011). Our observation also goes in consonance with another report where increase in temperature from 25°C to 37°C resulted in 26.05% to 98.13% naphthalene degradation after 72 h. This may be due to an increase in PAHs solubility and bioavailability with an increase in temperature and thus could be the reason behind our observation (Tirkey *et al*., 2021). Our finding is in agreement with a report on degradation of 4-chloroaniline (4-CA). (300 mg/l) was completely degraded by *Thauera* sp. M9, when grown at 30°C (Kumar *et al*., 2020).

HPLC analysis. The HPLC of abiotic control (2-NS) and samples treated with isolate C2 were compared (Fig. 7). The 2-NS showed a retention time at 11.857 minutes with height of 1266, whereas the treated sample showed same retention time but with significantly less height of 8.4, indicating to approximately 99.33% degradation of 2-NS by C2.

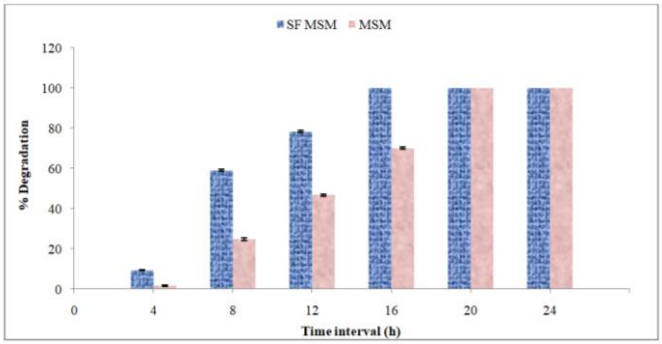


Fig. 1. Effect of two different medium on the degradation efficiency of isolate C2.

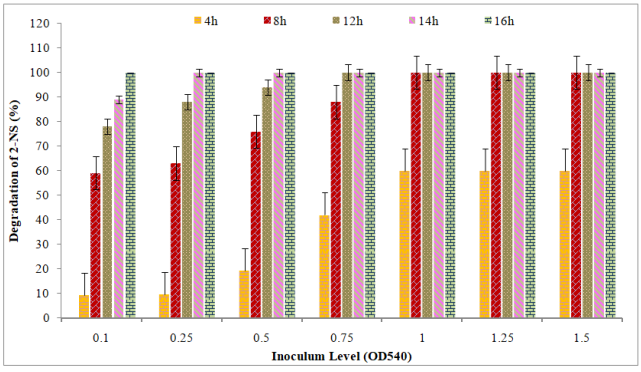
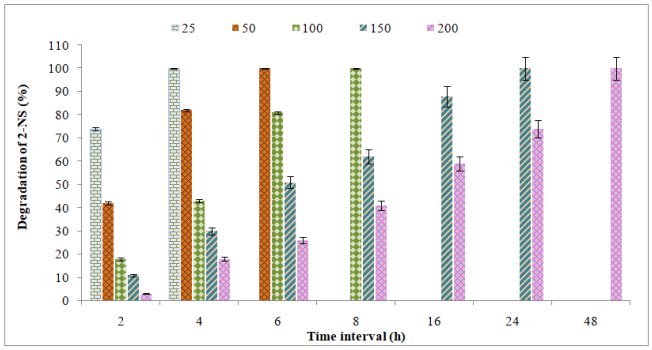
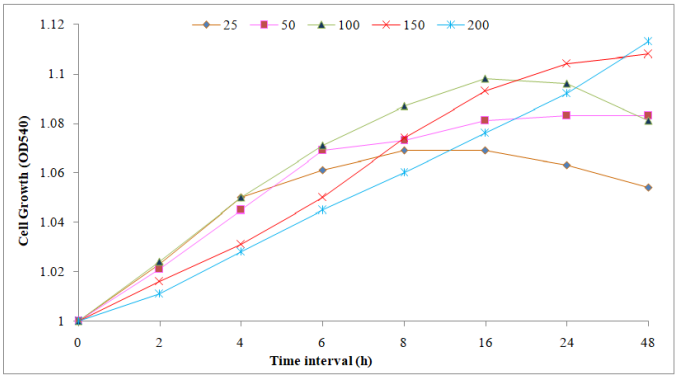


Fig. 2. Effect of addition of different inoculum level on the degradation of 2-NS (100mg/L) by isolate C2.



(A)



(B)

Fig. 3. Effect of addition of different concentrations of 2-NS on (A) Cell Growth and (B) degradation potential of isolate C2.

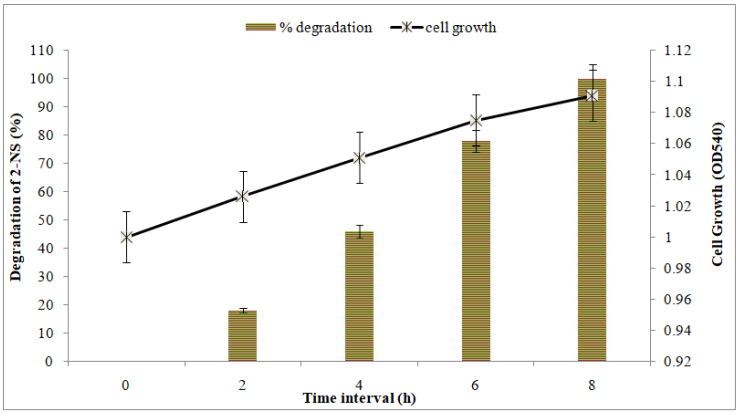


Fig. 4. Effect of different time interval of incubation on the degradation potential and Growth of isolate C2.

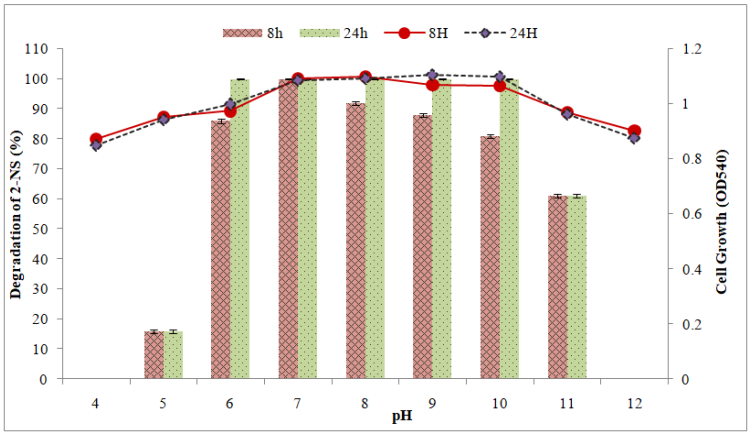


Fig. 5. Effect of initial pH of the growth medium on the degradation potential and Growth of isolate C2**.**

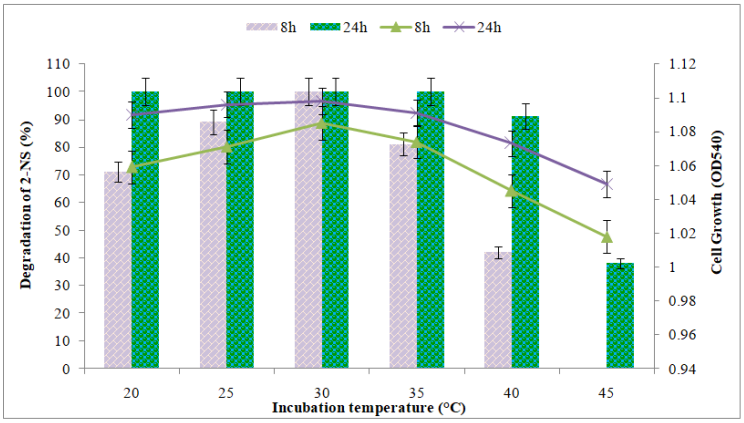
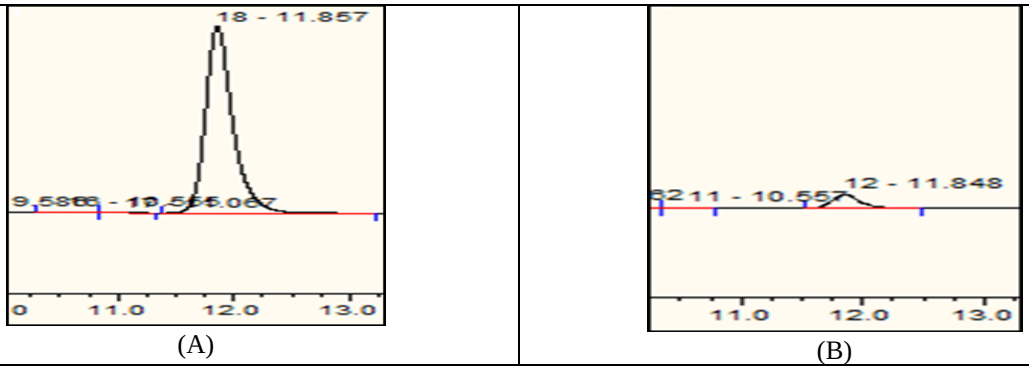


Fig. 6. Effect of different incubation temperature on the growth and degradation efficiency of isolate C2.



(A) (B)

Fig. 7. The HPLC analysis of A) 2-NS and graph (B) indicated the 100% degradation of 2-NS by isolate C2 within 8 hours under aerobic conditions.

CONCLUSIONS

This study thus highlights the significant effect of various physiochemical parameters on the degradation potential of isolate C2. The isolate exhibited an enhanced degradative capacity (100 mg/L of 2-NS in 8h) under these optimized conditions and was able to use 2-Naphthalene sulfonate as a sole source of carbon and energy. Further, the HPLC analysis of transformed sample (at 8h) revealed the complete degradation of 2-Naphthalene sulfonate**.** Therefore, the study concludes that the polluted sites could act as a source for isolation of indigenous potential degraders which could assist the scientific community to tackle the environmental haphazard created by such persistent organic pollutants.

FUTURE SCOPE

Future scope of the study includes identification of this potential degrader (isolate C2) and thereby explore its activity against other persistent organic pollutants, especially 2-NS- related naphthalene sulphonic acids. Further, the degradative pathway used by the isolate could also be elucidated using enzyme assays and spectroscopic analysis. In addition, the shake flask studies could be scaled up to lab scale bioreactor studies and testing the potential of the isolate to treat simulated effluent containing the given xenobiotic compound.

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Conflict of interest. None.

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