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Biodegradation of sulfanilic acid using *Bacillus cereus* AAA2018 from textile industry effluent contaminated soil

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Biodegradation of hazardous aromatic compounds is emerging as a potential tool for reduction of environmental pollution due to their high toxicity and complex synthetic nature. In this study textile effluent was used as a microbial source for aerobic degradation of sulfanilic acid. Two adaptation techniques were followed to maximize uptake of sulfanilic acid, provided as a carbon and nitrogen source. The continuous enrichment and acclimatization media techniques were carried out for 20 days, respectively and both samples were screened for better degradation efficiency. The isolates were found to be similar to the colonies obtained from effluent. Sulfanilic acid degrading organism was identified as *Bacillus cereus* AAA2018 using 16S rRNA sequencing. Similarly, study was done for fungal strain *Aspergillus japonicas*. The bacterial strain showed subsequent reduction of sulfanilic acid at minimal salt concentration, whereas *A. japonicus* showed very little degradation efficiency comparatively. The comparative study of biodegradation capacity of sulfanilic acid was confirmed using GCMS in which product degradation profile of both bacterial and fungal strains included Dimethyl sulfoxide (DMSO) and Phenol 2,4-bis(1,1-dimethyethyl) which gives an explanation for decrease in growth of bacterial culture as DMSO act as strong antibacterial agent.

Keywords: 16sRNA sequencing, Acclimatization, Biodegradation, Continuous enrichment, Dye industry, GCMS analysis, Textile effluent

Industries utilize different classes of chemicals in their process which are let outside in the environment untreated[1]. Among various industries, textile industries contribute a larger proportion of effluent consisting of organics such as polyvinyl alcohol (PVA), carboxymethyl cellulose and polycyclic hydrocarbons (PCH)[2,3]. Improper disposal of these wastes is the root cause for the settlement of azo and nitro compounds in the aquatic ground basin yielding potential carcinogens[4]. The functional groups present over these chemicals are mostly amine-NH_2, sulfonate-SO_3H and carboxyl-COOH[3]. These compounds are difficult to mineralize due to structural complexity and synthetic nature[5,3]. The characteristic study of these effluents shows around 200-1000 mg/L of chemicals with sulfonate groups[6].Sulfonated compounds are largely used in textile industries as dyes which show higher resistance to biodegradation under normal condition[7,8]. These chemicals are reported with major cause of chronic liver damage when consumed by the organisms. Thus, the present study shows an accumulation of the wastes which can be treated with microorganisms as an effective way to remove these chemicals and to reduce their toxic nature[3,9,10]. The process involves the stimulation of the degrading capabilities of microorganisms under aerobic condition, resulting in total mineralization[4]. Only very few microbes can utilize these compounds as source of carbon energy since substrate inhibition of growth at higher concentration, poses a critical issue in the biodegradation process[11,12]. Thus the microbial cells are made adaptable to higher substrate concentration using continuous enrichment and acclimatization techniques, in order to screen various microbes with active degradation ability[13,14]. Acclimatization technique exposes the microbes to gradual increasing concentration of chemical compounds which involves a stepwise addition of substrate doses at a constant concentration[15,12]. Similarly, continuous enrichment media expose the microbes to gradual increment of concentration in a

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series of flasks inoculated at regular intervals. Both the above-mentioned techniques indicate a closed culture system with mixed population, supplied with an exhausting specific limiting nutrient. This selective factor involves removal of cells of all species present, except the population in the media which prevails in the condition provided. Therefore, the techniques increase the capacity of the organisms to yield a secondary population with a particular organism having high predominance or degradation efficiency[15,16].

Many fungal strains have reported with successful degradation of the effluent compounds among which *Aspergillus japonicus* have larger enzymatic production for reduction of more complex structure such as tannic acid[17]. Similarly, *Bacillus* sp. strains are mostly recognized and characterized for cleavage of the polycyclic hydrocarbons which are otherwise toxic, carcinogenic, mutagenic or teratogenic to organisms. Some of the species viz. *Bacillus firmus* are studied for bioremediation process, and *Bacillus cereus*, *Pseudomonas Paucimobilis* are known for their degradation ability of phenol and other aromatic compound[12,18]. Here, we attempted sulfanilic acid as carbon as well as nitrogen source to bacterial colonies isolated from effluent contaminated soil, using two adaptation techniques to isolate a particular species. Further, we characterized their degradation ability and also did GCMS analysis.

Materials and Methods

Bacterial culture and growth conditions

Around 0.5 g of weighed effluent contaminated soil sample, collected from a textile industry near Tirupur, was enriched in 100 mL of Luria Bertani (LB) broth, incubated overnight at 37°C containing flucanozole. The culture was then serially diluted and plated on LB agar to obtain different bacterial strains. Selective media was prepared consisting of sulfanilic acid as a sole carbon source with initial concentration of 50 mg/L with trace salt solution (TSS) composed of $CaCl_2$ 20 mg/L; $MgCl_2$ 80 mg/L and K_2HPO_4 200 mg/L, to which 50 mg/L of further salt concentration was successively added with respect to the technique used. In acclimatization (A) media technique, the cultures were acclimatized to sulfanilic acid by exposing them to periodic addition with 50 mg/L of concentration[18,19]. Similarly for continuous enrichment (CE) media technique, after 3 days of initial incubation, the sample was transferred as 5% inoculum to the flask containing 100 mg/L. A series of flask were inoculated at an interval of 3 days where in the content of sulfanilic acid was increased, until it reached a maximum concentration of 350 mg/L. For the inoculum transfers, it was all done in exponential phase[16,20]. As above a comparison study was accomplished using a selected fungal strain, *Aspergillus japonicus* enriched in a potato dextrose broth for 7 days at 37°C. They were acclimatized to subsequent periodic addition of sulfanilic acid.

Identification of bacterial isolates

Each sample culture was checked for its decrease in the turbidity and was plated on to LB agar with serial dilution to isolate the colonies. To identify the predominant bacteria able to degrade the sulfanilic acid at higher salt concentration, different bacterial colonies were picked up from the plated agar plates from both A and CE samples and the bacterial DNA was isolated. About 5 µL volume of isolated bacterial DNA was amplified in 25 µL PCR reaction solution consisting of 1.5 µL of both forward and reverse primers, 5 µL of de-ionized water and 12 µL of Taq master mix. The 16S rRNA genes were amplified using two sets of primers. The forward primer 8F (5' AGAGTTTGATCCTGGCTC 3') and reverse primer 1541R (5' AAGGAGGTGATCCAGCCGCA 3'). The obtained target DNA was purified with ethanol precipitation protocol and was subjected to electrophoresis in an ABI 3730X1 sequencer (Applied Biosystems). The resulted amplified sample was then aligned to 16S rRNA sequences extracted from National Centre of Biotechnology Information (NCBI) database by BLASTN 2.8.0+ program[21].

Characterization of growth and degradation

One mL of A, CE and fungal sample (F) were collected at a periodic interval of 3 days. The growth and degradation of the isolates was characterized using Ultraviolet Visible (UV-Vis) spectrophotometer at 600 and 290 nm up to 20 days of accounting, respectively. The observed values were plotted as growth and degradation curve. The degradation efficiency was determined using Bratton Marshall (BM) assay at pH 7.0 with addition of 0.05 Mm of Fe^{2+} [17]. A calibration curve was plotted for the working standard of sulfanilic acid ranging from 0.5-2.5 mL. About 1.0 mL of unknown sample was subjected to 2.5 mL of hydrochloric acid and 0.5 ml of 0.4% sodium nitrite was added. Vortex it for 5min until there is liberation of nitrous acid. This was followed by addition of 1.0 mL 0.5% ammonium sulphamate and left for 2 min. One mL of N-(1-

napthyl) ethylene diamine dihydrochloride reagent was added and mixed well. The volume was then made up to 7.5 mL with distilled water. The pink color was developed by diazotization and the absorbance was measured at 540 nm[22-24].

Characterization of the degradation profile using GCMS analysis

GCMS analysis was performed in an analytical column HP-5 MS 5% phenyl methyl silox capillary column with a film thickness of 30 m×250 µm×0.25 µm. The injector temperature was 250°C and the sample injection volume was 1.0 µL. The flow rate of the helium carrier gas was maintained at 1.0 mL/min. The oven temperature was programmed to 50°C for 2min, increasing up to 270°C with a gradual increment of 10°C /min which was then held for 10min at 270°C. The substance analysis was compared with data from NIST MS 2011 Library using Agilent mass Hunter software[25,26].

Result and Discussion

Bacterial strains from different samples

Two morphologically different strains were obtained from the effluent contaminated soil samples, named ES_1 and ES_2. The pigmentation of $ES_1$was colorless transparent irregular colony and ES_2 was white colony. Morphologically similar strains ES_1 were observed in Continuous Enrichment (CE) and Acclimatized culture (A). Thus, only one out of two strains, present in the effluent contaminated soil sample has the ability to tolerate higher substrate concentration and could utilize sulfanilic acid as carbon and nitrogen source.

Growth and Degradation curve of bacterial and fungal strain for different adaptation techniques

The mixed culture inoculated in the respective continuous enrichment and acclimatization media exhibited a maximum growth rate at a critical concentration of 100 mg/L of sulfanilic acid. The respective standard growth curve was plotted for the absorbance value obtained from sample at a regular time interval of 3 days.

In Fig. 1, the bacterial growth curve of CE sample depicts an ideal growth curve with exponential and stationary phases. The turbidity of the medium increases gradually and took a high peak after 5 days marking active log phase. After the active log phase the medium showed gradual deceleration in its bacterial count and further a stagnant improvement in its growth. This represents the species stimulation to

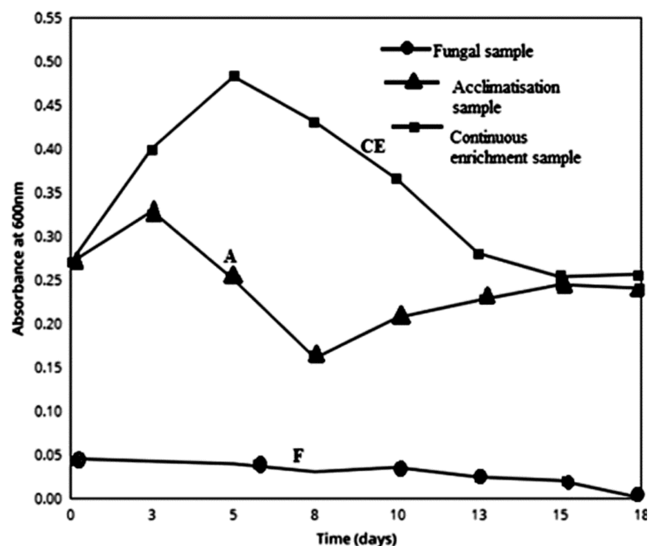

Fig. 1 — Growth curve of acclimatization sample (A), Continuous Enrichment sample (CE) and Fungal sample cultures (F)

tolerate the given salt concentration with its ability to adapt in the given condition. Similarly the bacterial growth of A sample shows a higher turbid medium at a concentration of 100 mg/L. Further, it accounts screening of the bacterial species with higher tolerance to the incremental salt concentration as a limiting substrate. It marks a decelerating phase after 3rd day in the graph where the species are screened for their adaption and tolerance. It is noteworthy that sulfanilic acid acts as an antimicrobial agent at higher concentration which makes the utilisation of the mentioned compound as sole source for energy remains a difficult task. Thus after a sudden deceleration, the bacteria undergoes a genetic drift such that it produces a secondary culture to withstand higher salt concentration and similarly reflects as increase in the turbid of medium. When compared to the growth of the fungal strain *A. japonicas* (sample F) which exhibits its maximum growth only at the initial minimal salt concentration and shows its inability to adapt to the given conditions. As the concentration gradually increases the growth takes an exponential peak, a little less than its maximum turbidity, thereby evolving to adapt to the increasing salt concentration. But after the critical concentration of 100 mg/L, the growth gradually decreases with increase in the concentration.

The degradation profile of the microbial culture plotted depicts a logistic regression of the concentration of sulfanilic acid using UV- visible spectrophotometer in Fig. 2 and found to be similar to the degradation pathway proposed by Perei *et al.*[17] in

which initial desulfonation was observed in the cell extract by the di- or mono-oxygenase activity liberating the sulfonic group prior to the ring cleavage. As mentioned, in similar to the growth curve of CE sample observed, the maximum breakdown of the component occurs during the 5th day of its growth (exponential phase). The breakdown remains stagnant as of growth after the maximal degradation of the component after 8th day. Parallel studies for degradation curve of the bacterial culture in sample A depicts similar breakdown of the component as in sample CE and undergoes a wavering exponential regression. The wavering plot shows the adaptation and evolution of secondary culture with its enhanced ability to degrade the component due to the role of oxygenase. Comparative study of the fungal degradation shows a decreasing concentration profile of the sulfanilic acid which relates only a slight deviation from the initial concentration. The fungal strain shows a very little chance of adaptation to the growth condition prevailing with incremental salt concentration.

The isolates were examined for their degradation potential or ability using BM reagent. Different concentration of the sulfanilic acid present in the sample at different intervals was checked. The supernatant collected from A, CE and F samples were diazotized with BM reagent to produce dark pink colour. The final salt concentration was determined using the calibration curve plotted with the working standard which was computed to obtain the degradation efficiency. The concentration of sulfanilic acid was greatly reduced up to 74.8 and 73% in A and CE, respectively from the initial concentration but the fungal degradation contributed only 10%.

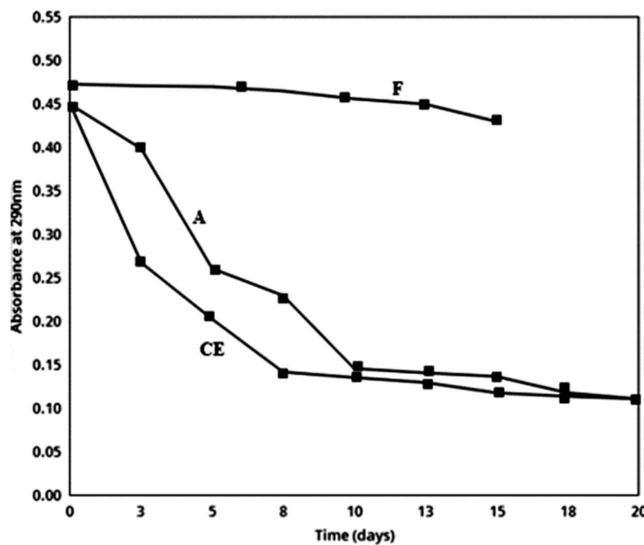

Fig. 2 — Degradation curve of acclimatization sample (A), Continuous Enrichment sample (CE) and Fungal samples(F)

Concentration and screening of 16S rRNA clone library and phylogenetic tree analysis

Bacterial 16SrRNA genes were blast using NCBI blast similarity search. The phylogeny analysis of the query sequence with the closely related sequence was performed followed by multiple sequence alignment and the blast results were obtained (Fig. 3). The obtained 997 bp amplified target was sequenced as follows:

CCTTAACTACATGCAGTCGAGCGAATGGATT
AAGAGCTTGCTCTTATGAAATTAGCGGCGGAC
GACGTGAGTAACACGTGGGTAACCTGCCCATA
AGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATAACATTTTGAACCGCACGGGTCGA
AATTGAAAGGCGGCTTCGGCTGTCACTTATGG
ATGGACCCGCGTCGCATTATCTAGTTGGTGAG
GTAACGGCTCACCAAGGCGACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGAACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCT
GACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGA
ACAAGTGCTAGTTGAATAAGCTGGCACCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTAC
GTGCCAACAGCCGCGGTAATACGTAGGTGGCA
AGCGTTATCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGTGGTTTCTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGAAAA
CTGGGAGACTTGAGTGCAGAAGAGGAAAGTGG
AATTCCATGTGTAGCGGTGAAATGCGTAGAGA
TATGGAGGAACACCAGTGTCGAAGGCGACTTT
CTGGTCTGTAACTGACACTGAGGCGCGAAGCG
TGGGGAGCAACAGGATTAGATCCCTGGTAGTC

Fig. 3 — Phylogenic tree rendering of *Bacillus* sp.

Table 1 — Components present in the medium after degradation in both CE & AS sample (%)

Name of the compound	Peak Height	Conc. (%)
Dimethyl sulfoxide	76496	23.125
Phenol 2,4-bis(1,1-dimethylethyl)	17984	7.71

[CE, Continuous Enrichment sample; AS, Acclimatization sample]

Table 2 — Components present in the medium after fungal degradation (%)

Name of the compound	Peak Height	Conc. (%)
Tetraacetyl-d-xylonic nitrile	282307	14.222
Dimethyl sulfoxide	49237	2.481
Cyclopent-2-enone 5-allyl-3-benzyl amino-2,5-dichloro-4,4-dimethoxy	15336	0.773
Phenol 2,4-bis(1,1-dimethylethyl)	104191	5.249

ACGCCGTAACGATGATGCTAGTGTTAGAGGTT
TCCGCCCTTAGTGCTGAAGTTACGCATTAAGCC
TCCGCCTGGGGAGTACGGCCGCAGGCTGAACT
CAAGGAATTGACGGGGCCCGCACAGCAGTGGA
GCAGTGTTTATTCGAACCACGCGAAAAACTTA
CAGGCCTGACTCCTCTGACACCCTAAGATAGG
CTTCCCTTCGGAACAAATGACAGGGGTGCAGG
ATGTCGTC

The derived aligned sequences were similar to the strain *Bacillus cereus* and was confirmed using the phylogeny tree rendering. The above 16S ribosomal RNA partial sequence nucleotide analysis was submitted to the NCBI and the accession number was assigned to be MH109322 of version MH109322.1. The organism isolated was identified as *Bacillus cereus* strain *AAA2018*.

GC-MS analysis

The GCMS analysis was done to identify the degradation products of the samples obtained during their growth with respect to the utilization of sulfanilic acid as carbon and energy source. The product profile obtained for bacterial strains involved the production of two predominant components- dimethyl sulfoxide and phenol 2,4 bis (1,1-dimethylethyl) as depicted in Table 1. Comparison of the product profile with fungal strain involves the production of new synthetic compounds such as tetraacetyl-d-Xylonic nitrile and cyclopent-2-enone, 5-allyl-3-Benylamino-2,5-dichloro-4,4 dimethoxy which is tabulated in Table 2. The product profile also showed the bacterial degradation products dimethyl sulfoxide and phenol, 2, 4-bis (1,1-dimethylethyl) which gave us a wide assumption of the degradation of sulfanilic acid (Fig. 4).

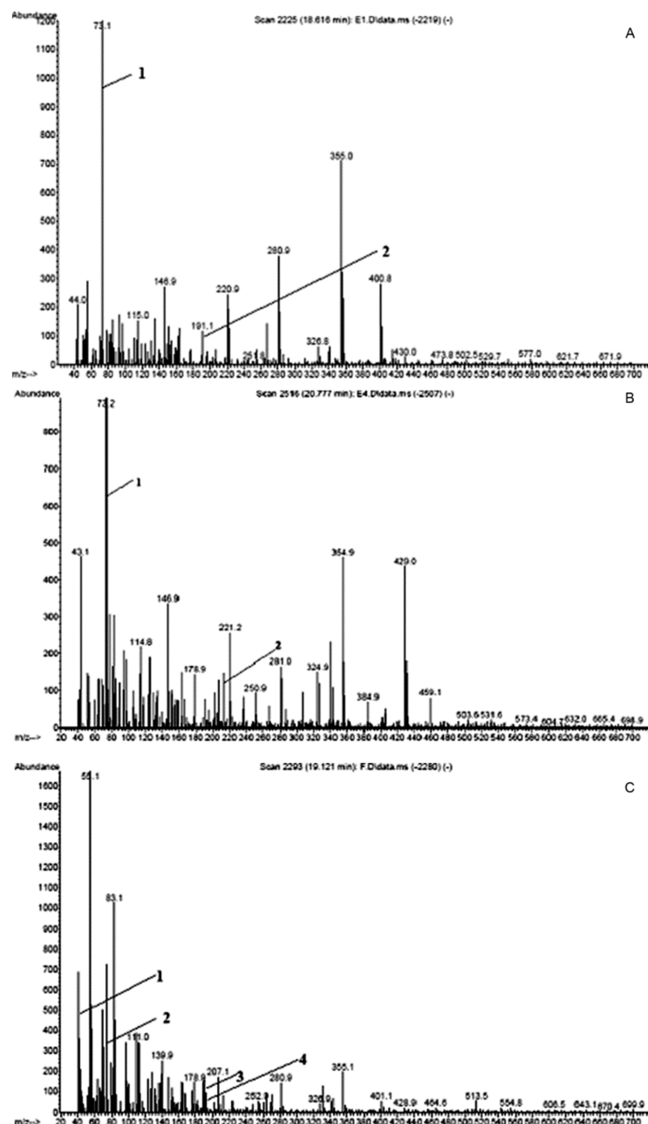

Fig. 4 — Mass chromatograms of degradation product profile of (A) Acclimatization sample (A); (B) Continuous Enrichment sample (CE); and (C) Fungal sample cultures (F)

Conclusion

The bacterial strain showed subsequent reduction of sulfanilic acid at minimal salt concentration, whereas *Aspergillus japonicus* showed less degradation efficiency as compared to bacterial strain. The product profile of fungal sample showed synthesis of various new compounds which cannot be obtained easily via natural or chemical synthesis, giving an acknowledgement for the genetic changes with respect to tolerance. The product degradation profile of both bacterial and fungal strains included DMSO and phenol 2,4-bis(1,1-dimethyethyl) which gives an explanation for decrease in growth of

bacterial culture after certain period of time, assuming that they only got adapted until the formation of secondary culture. Production of DMSO acts as an antimicrobial agent under high concentration. Both products can be utilized in many different fields, such as DMSO as an antimicrobial agent in medicines and phenol, 2,4-bis(1,1-dimethylethyl) can be used as an active biosurfactant. Hence, in a continuous or fed batch system where there is a continual removal of DMSO, intrinsic medium factor can also be eliminated as a growth inhibitor, allowing the microbes to fully utilize their efficiencies.

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