

DEGRADATION OF PHENOL USING A PHB PRODUCING *BACILLUS SUBTILIS* SP3**Indu Chandrasekharan Nair* and Sukanya Prakash**

Assistant Professor, Department of Biotechnology, SAS SNDP Yogam College, Konni,
Pathanamthitta, Kerala, India.689691.

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Corresponding Author*Dr. Indu****Chandrasekharan Nair**

Assistant Professor,
Department of
Biotechnology, SAS
SNDP Yogam College,
Konni, Pathanamthitta,
Kerala, India.689691.

ABSTRACT

The bacterial isolate from soil grown in the designed synthetic medium with phenol as the sole carbon and energy source could degrade phenol completely. The isolate showed fast acclimatization in the minimal medium. Polyhydroxy butyrate (PHB), an alternative to synthetic plastic was also extracted from the same bacterial isolate. The PHB granules from the newly screened strain designated as sp3 were visualized by atomic force microscopy. 16S rRNA analysis of the bacterial isolate established the identity of the strain as *Bacillus subtilis* sp. The effect of pH and incubation time under phenol stress were optimized for maximum growth. It was observed that the optimum pH was 7, incubation time was 24 hours and phenol concentration was 12 mM. The degradation was analyzed by Fourier Transform Infra-Red spectroscopy. This strain could also be successfully used for the

laboratory scale treatment of paper mill effluent containing phenolic components. The wastewater showed a considerable COD reduction of 64% after 24 hours of treatment.

KEYWORDS: Phenol degradation, *Bacillus subtilis* sp3, Poly Hydroxy Butyrate, Fourier Transform Infrared Spectroscopy, Chemical Oxygen Demand.

INTRODUCTION

Phenol and its derivatives are toxic and are classified as hazardous materials.^[1] Enlisted by Environmental protection Agency as a priority pollutant phenol provides far ranging effects if it gets into the ecosystem. Phenol reaches the environment from both natural and industrial resources. Effluents originated from oil refineries, pulp and paper manufacturing plants, steel,

and pharmaceutical industries contain phenol and phenolic compounds. On reaching the ecosystem they provide stress to the biotic components. Continuous and short term exposures are deleterious since it leads to restricted growth rate, lower fertility and metabolic abnormalities. As a disinfectant, phenol can inhibit the growth of microorganisms including bacterial, protozoan and algal community leading to interruption of food chain. Phenolic components affect skin and exposure produces lesions on fishes.^[2] Waste water containing phenol and its derivatives must be treated to prevent serious ecological risks.

Various approaches are reported to manage the treatment of phenolic contaminants in water. Microorganisms are promising candidates for biodegradation and remediation. Phenol can be used as the sole carbon source by many bacteria and fungi which have evolved mechanisms to tolerate and utilize phenol. Generally such microorganisms show vast diversity in their biochemical properties and can decompose many complex organic substances including aromatic compounds. In phenol biodegradation, enzymatic break down of aromatic rings is reported to be effected through meta or ortho cleavage pathway.^[3] The main group of enzymes involved are phenol hydroxylases and phenol oxidases which have been isolated and characterized from both fungi and bacteria.

This potential of microbes can be employed in bioremediation procedures. Chemical, physical and biological methods are used separately and in combination for the treatment of phenol and other aromatic compound contamination. Biological treatment has shown to be economical, practical and most versatile approach as it leads to complete mineralization of phenol.^[4] In the present study, bacteria from a detergent contaminated soil were screened for its phenol degradation ability with a view to utilize them for bioremediation. The bacterium was also found to accumulate Poly Hydroxy Butyrate (PHB) which is an alternative for synthetic plastic. The main advantage of PHB includes their superior environmental qualities. Poly hydroxyl butyrate has very good barrier properties; it is water insoluble and is susceptible to microbial degradation.

MATERIALS AND METHODS

2.1. Sample collection

Detergent contaminated soil samples from four different regions of Pathanamthita District, viz; Edathitta, Elakolloor, Pathanapuram and Konni, Kerala, India were collected for the primary screening of phenol degrading bacterial isolates.

2.2. Screening for phenol degradation

Screening for phenol degrading bacterial isolates was done from the above soil samples as follows: 10 g soil from each sample was mixed with 100 ml of sterile distilled water and allowed to sediment for a while. The supernatant was used as inocula for serial dilution. From each dilution 1 ml was inoculated to 100ml sterile peptone water containing 1mM phenol. The conical flasks were incubated overnight at 37⁰C on a rotary shaker at an rpm of 300.

Bacterial samples obtained from primary screening were used as inocula for enrichment. 10ml of 24 hour old cultures in peptone broth were centrifuged at 5000 rpm for 10 minutes and corresponding pellet was suspended in 1ml physiological saline. Each of the bacterial suspensions were inoculated into 100 ml Mineral Salt Phenol Medium(MSPM) with the following composition: 1g KH₂PO₄, 1g (NH₄)₂SO₄, 0.50g MgSO₄.7H₂O, 1mg CaCl₂ in 1 litre at pH 7. Phenol, the sole carbon source, was added from the stock to a concentration of 4mM after autoclaving the medium. The medium was shaken well and incubated overnight at room temperature in a rotary shaker at 300 rpm. A consortium of microorganisms consistently growing in the basal medium at 4 mM phenol concentration was selected and was subjected to further isolation.

2.3. Isolation of pure colonies from the consortium

Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4⁰C. Out of the three pure isolates one showing fastest growth under normal conditions in mineral salt phenol medium was selected for further studies.

2.4 Identification of microorganisms

Identification of the selected phenol degrading bacteria was done by conducting various morphological and biochemical tests as per Bergey's manual of Systematic Bacteriology.^[5]

Identification by 16SrRNA gene analysis

The molecular characterization procedures involves Isolation of DNA, 16s rRNA analysis by amplification through polymerase chain reaction followed by sequencing and BLAST analysis. 16s rRNA genes are amplified by PCR using universal primers.

Forward primer: F- (5'-AgA gTT TgA TCM Tgg CTC-3'),

Reverse primer: R- (5'-AAG gAg gTg WTC CAR CC-3').

Other requirements include DNA 5ng/ml, PCR ready mix(Merck) made upto a volume of 25 microlitre. PCR Programme includes 25 cycles of: 95⁰C, for 30S, 55⁰C, for 30S, 72⁰C for 30S, 72⁰C for 5 minutes.

Bacterial isolates were tested for species identity using the 16s rRNA sequencing method.^[6] Sequence analysis was performed with sequences in the National Centre for Biotechnology (NCBI), USA database using Basic Local Alignment Search Tool (BLAST) for Nucleotides and a phylogenetic tree was constructed.

2.5. Phenol degradation studies

Phenol estimation of the various samples were done through spectrophotometric estimation.^[7] Optimum concentration of phenol utilized by bacteria and inhibitory concentration of phenol on growth were also found out.

2.6. Staining methods for PHB granules

a. Sudan black staining

Sudan black staining was done to identify the cells producing PHB. 0.75 g Sudan black was dissolved in 10 ml ethanol and the solution was filtered using Whatman No 1 filter paper. A thin smear was prepared from the suspension on a clean dry microscopic slide by heat fixing. Sudan black stain was flooded over the smear and kept for 5-10 minutes. The slide was washed with distilled water, decolourized with xylene and counter stained with saffranin for 10 seconds. The slides were washed with water, dried and observed under oil immersion objective of compound microscope with magnification 100X.^[8]

b. Nile blue a staining

The bacterial cells in MSPM medium were subjected to centrifugation at 5000 rpm for 30 minutes. The supernatant was resuspended in 1 ml sterile saline. This was used to prepare the smear. The heat fixed smear of the bacteria was stained with 1% aqueous solution of Nile blue A. This solution was heated for 10 minutes at 55⁰C. The slide was washed with tap water and was placed in 8% aqueous acetic acid solution for 1 min.^[9] After washing the slide was observed under atomic force microscope (WIT ec alpha 300 RA) at sophisticated and test instrumentation facility, M.G. University, Kottayam.

2.7 Growth curve in nutrient broth and MSPM

The organism was cultured in 100 ml in nutrient broth and growth was detected in every hour after the inoculation in terms of turbidity at 650 nm using a spectrophotometer for 30 hours. The same was also performed with MSPM containing optimum phenol concentration.

2.8. Extraction of PHB

Cells were collected by centrifugation at 7000 rpm for 10 minutes. Pellets were washed with phosphate buffered saline, air dried and weight taken. Chloroform and 4% sodium hypochlorite were added to the cell pellet in the ratio 1:1/mg of pellet weight. After overnight incubation at 30°C the pellet was centrifuged and the bottom phase containing PHB was transferred to another fresh tube and the volume measured. Then 5X volume of a mixture of methanol and water (7:3v/v) was added. After centrifugation the precipitate was collected and washed with water and isopropanol.^[9]

2.9. Fourier Transform Infrared analysis

The biodegradation of phenol and also the accumulation of PHB were confirmed by Fourier Transform Infra-Red spectroscopy. The ether extracts of both uninoculated and inoculated MSPM were subjected to FT/IR analysis.

2.10. Optimization of pH and incubation period for maximum growth

pH

100 ml aliquots of the mineral salt phenol medium prepared with pH varying from 2 to 12 were taken, inoculated with the bacterial suspension and incubated at room temperature on a rotary shaker at 300 rpm. Growth of the culture was observed after 24 hours.

Incubation period

100 ml aliquots of the mineral salt phenol medium taken were inoculated with the bacteria and incubated for various time intervals on a shaker at 300 rpm. Turbidity of the culture measured at 600 nm in every hour after inoculation and was plotted against various incubation time.

2.11. Treatment of paper pulp waste water

Waste water collected from the paper pulp industry was diluted by 4 times and the phenol concentration was measured. 100 ml of waste water was inoculated with 1ml(1OD) cell suspension and incubated overnight at room temperature on a rotary shaker at a speed of

300rpm. Phenol content and COD of the waste water sample were analyzed before and after incubation as per the procedure of ASTM.^[11]

3. RESULTS AND DISCUSSION

3.1. Screening for Phenol degradation

The microbial biodiversity is vast to be identified and the biochemical versatility of microbes is yet to be exploited. The bioremediation approaches focus on this potential and intend to screen out organisms which can decompose toxic chemicals through unique metabolic pathways mediated by enzymes. In the present study *Bacillus subtilis* sp designated as sp3 was confirmed to utilize phenol which is a common aromatic contaminant in many industrial effluents.

The soil samples from detergent contaminated areas of four different places viz. Edathitta, Elakolloor, Pathanapuram and Konni, situated in the district of Pathanamthitta, Kerala, India were subjected to screening and the samples from all the different regions showed the presence of phenol tolerating bacteria. 12 isolates were obtained from primary screening and when subjected to enrichment, bacterial isolates in the sample from Edathitta showed maximum efficiency in phenol degradation. The mineral salt phenol medium used for screening contained phenol at 4mM concentration as the sole source of carbon. The sample was found to be a consortium on quadrant streaking and the components were subjected to isolation. Enrichment is an apt method to screen out bacteria capable of utilizing complex substances. The gradual increase in the concentration of phenol in the MSPM leads to the isolation of efficient strains involved in phenol degradation.

3.2. Isolation and identification of microorganism from the consortium

Three types of colonies were identified and are named as sp1, sp2 and sp3 which were selected on the basis of growth under stress induced by phenol. It is well known that certain groups of microorganisms produce poly hydroxy alkanoate granules under stress condition as reserve energy store.^[12] The strain SP3 from the consortium was identified as the fast growing and most efficient phenol degrading bacteria with PHB accumulation and was selected for further studies (Table 1). The identity was established through morphological and biochemical analysis of the isolate. 16S rRNA analysis is most dependable for characterization of bacteria as it provides a conserved regions in required rates. Phylogenetic tree was constructed and the bacterium showed maximum similarity to *Bacillus subtilis* and

was designated as *Bacillus subtilis* sp3. The sequence and dendrogram are given as Fig.1 and Fig.2.

Table 1: Secondary screening of the three selected isolates based on the percentage of phenol degradation obtained in MSPM during 24 hours of incubation.

| Designation of microorganism | Percentage of Phenol degradation |
|------------------------------|----------------------------------|
| sp 1 | 66.66±0.6 |
| sp2 | 54.40±0.4 |
| sp3 | 99.99±0.7 |

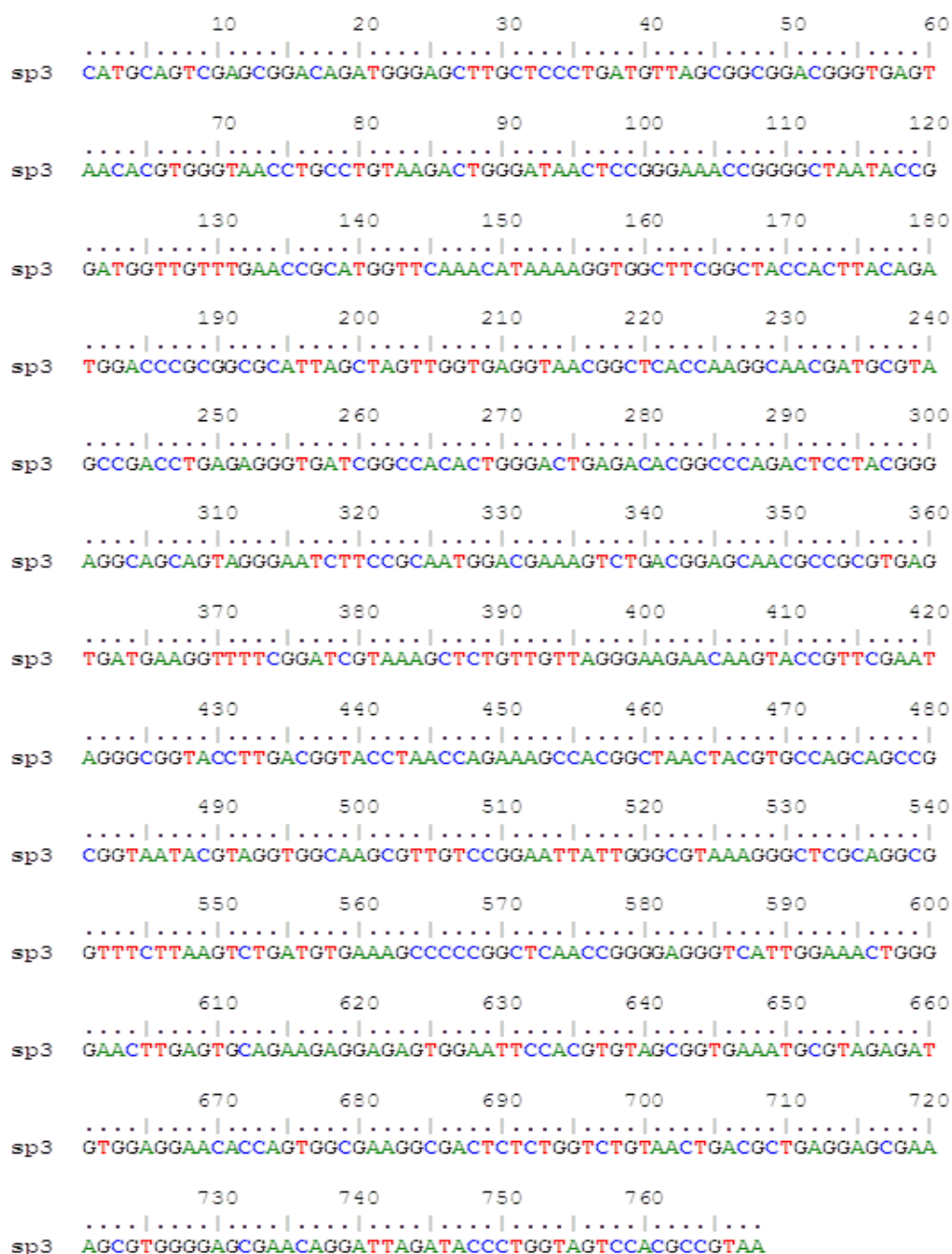


Fig 1: 16 S r RNA gene sequence of the selected isolate sp 3.

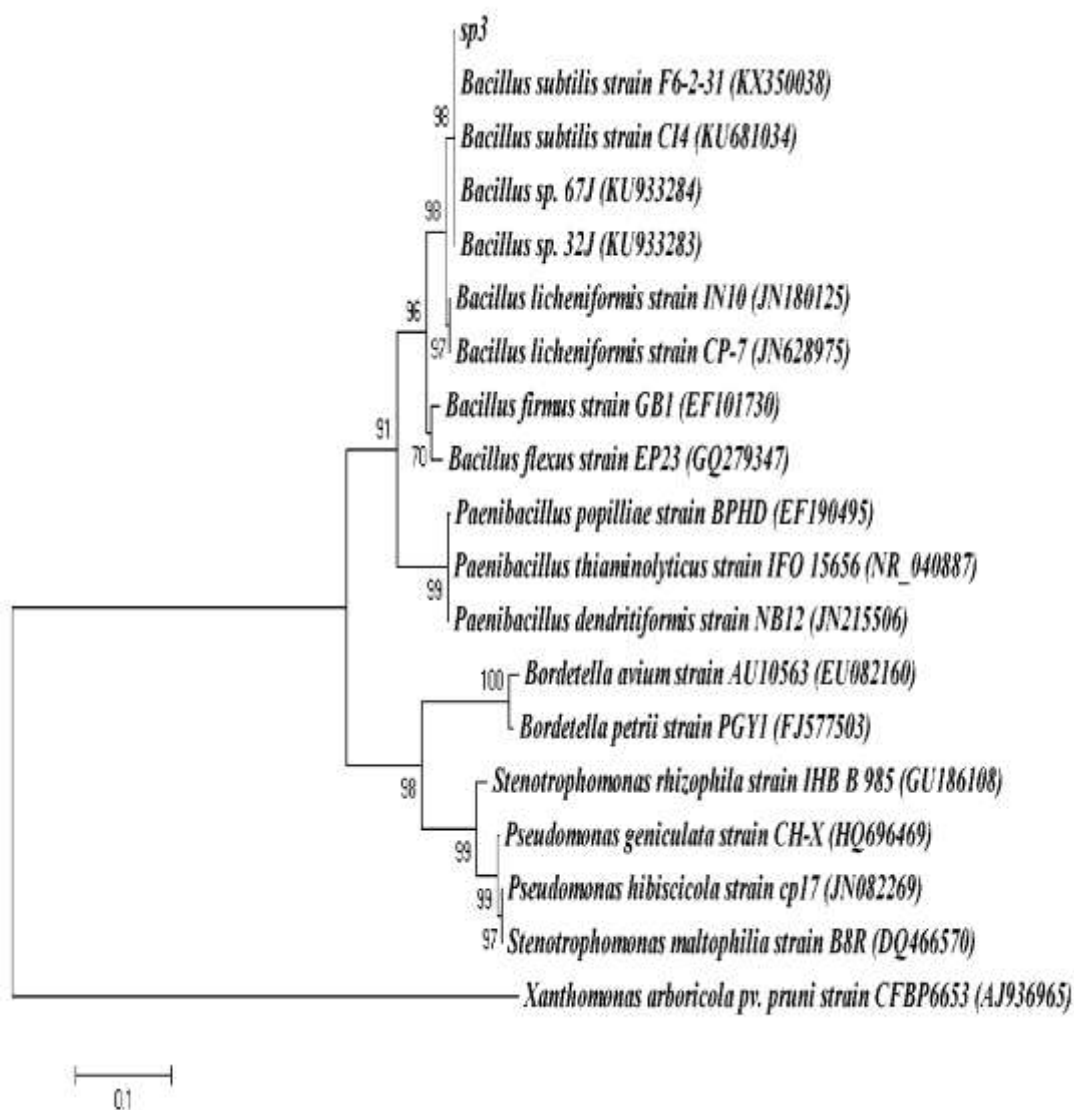


FIG 2: Dendrogram Of sp3.

3.3. Phenol degradation and PHB accumulation

Phenol degradation and intracellular PHB accumulation efficiency of the selected isolate is a unique property. The organism was capable of degrading phenol up to 30mM concentration. The rate of phenol degradation was found to decrease along with increase in concentration of phenol from 12mM concentration. There was no growth in 30 mM phenol concentration which could be considered as the inhibitory concentration of phenol to the selected bacterial isolate. Phenolic stress induces the production of PHA granules. In all the three isolates PHB granules were observed during sudanblack staining. The presence was again confirmed by Nile blue staining for PHB granules using Atomic Force microscopy (Fig.3). PHB is produced in the bacteria as a reserve food material and this can be extracted out from bacteria as they attain sufficient growth. This biodegradable PHB is an excellent alternative to synthetic

plastic. Scale up and optimization are needed to enhance the accumulation of PHB under phenol stress and also to enhance the amount extracted from the bacterium.

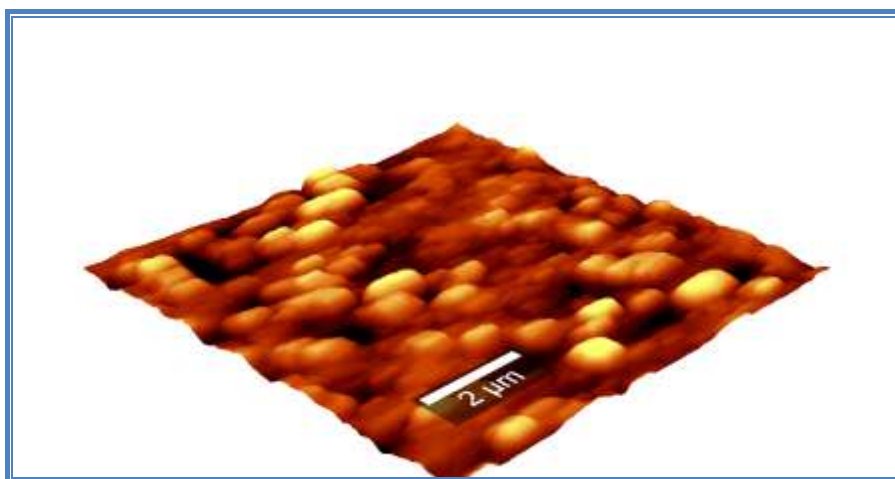


Fig 3: Atomic force microscopic image showing PHB accumulation in *Bacillus subtilis* sp3 grown in MSPM.

3.4. Growth studies of the selected isolate *Bacillus subtilis* sp3

Growth studies in nutrient broth was conducted to monitor the growth of the selected organism in a general nutrient medium and growth studies in MSPM was conducted to analyze the growth pattern of the strain in specific basal medium (Fig.4 and Fig.5) In MSPM, phenol is being provided as both carbon and energy source. When comparing the growth curves it was clear that in the MSPM the initial lag is well extended. In the stress condition the microorganism takes time to acclimatize with the condition. The increase in turbidity indicated the active growth of bacteria. After acclimatization the bacteria could exhibit fast growth in MSPM and reached the stationary phase at about 26th hour as in nutrient broth.

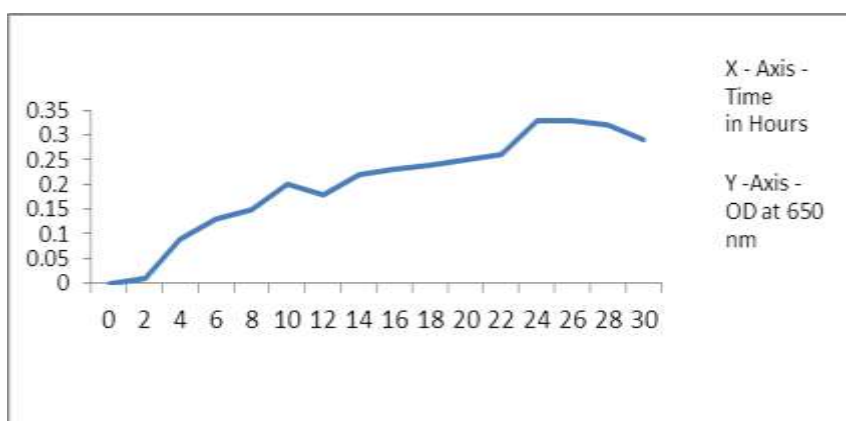


Fig 4: Growth curve of the selected *Bacillus subtilis* sp3 in nutrient broth.

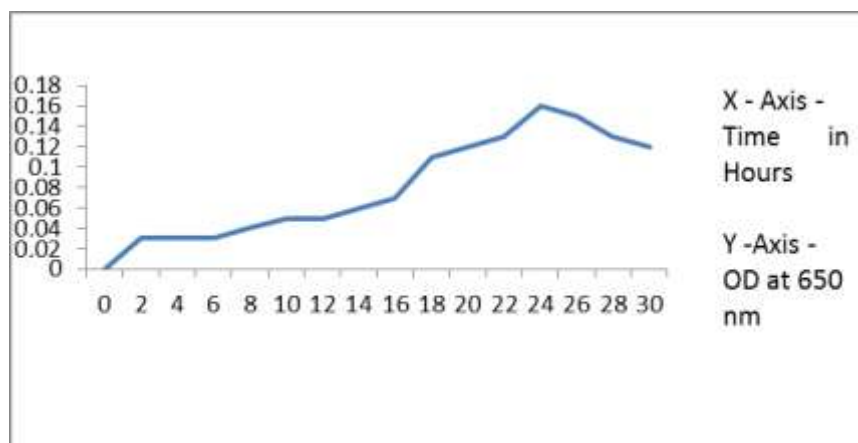
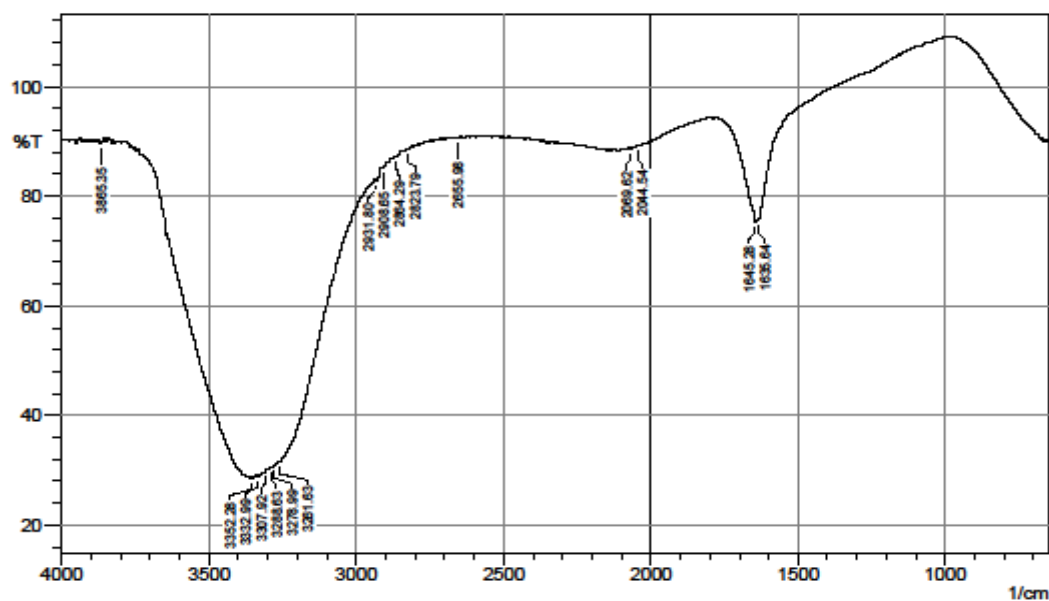


Fig 5: Growth curve of the selected *Bacillus subtilis* sp3 in MSPM.

3.5. FT/IR analysis of the mspm for confirming phenol degradation and PHB production

FT/IR analysis of the organic extracts of MSPM inoculated with *Bacillus subtilis* sp3 was carried out to trace the phenol degradation and PHB production after 24 hours of incubation. Fig.6 represented the control, ie the FT/IR of the uninoculated MSPM and the Fig.7 represented the sample ie MSPM inoculated with the selected isolate and incubated for 24 hours. The broadening of bands above 3000 cm^{-1} together with the new bands formed at 2700 cm^{-1} , 2300 cm^{-1} , nearly 2000 cm^{-1} represented PHB accumulation during phenol degradation. PHB is accumulated intracellularly in the form of inclusion bodies and may account for up to 90% of the cellular dry weight in many cases.

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| No. | Peak | Intensity | Corr. Intensity | Base (H) | Base (L) | Area | Corr. Area |
|-----|---------|-----------|-----------------|----------|----------|-------|------------|
| 1 | 1635.64 | 75.551 | 0.546 | 1637.56 | 1535.34 | 6.081 | 0.032 |
| 2 | 1645.28 | 75.349 | 1.074 | 1762.94 | 1639.49 | 7.91 | 0.042 |
| 3 | 2044.54 | 89.148 | 0.046 | 2046.47 | 1870.95 | 7.066 | 0.073 |
| 4 | 2069.62 | 88.765 | 0.126 | 2071.55 | 2056.12 | 0.788 | 0.006 |
| 5 | 2655.98 | 90.669 | 0.152 | 2665.62 | 2644.41 | 0.893 | 0.007 |
| 6 | 2823.79 | 88.554 | 0.27 | 2827.64 | 2812.21 | 0.793 | 0.01 |
| 7 | 2864.29 | 87.111 | 0.226 | 2866.22 | 2843.07 | 1.31 | 0.013 |
| 8 | 2908.65 | 85.187 | 0.264 | 2912.51 | 2883.58 | 1.9 | 0.013 |
| 9 | 2931.8 | 82.911 | 0.73 | 2937.59 | 2912.51 | 1.923 | 0.062 |
| 10 | 3261.63 | 31.438 | 0.379 | 3263.56 | 2939.52 | 82.4 | 0.679 |
| 11 | 3278.99 | 30.835 | 0.095 | 3280.92 | 3265.49 | 7.828 | 0.026 |
| 12 | 3288.63 | 30.592 | 0.092 | 3290.56 | 3280.92 | 4.947 | 0.009 |
| 13 | 3307.92 | 29.634 | 0.186 | 3309.85 | 3304.06 | 3.025 | 0.001 |
| 14 | 3332.99 | 29.041 | 0.098 | 3334.92 | 3321.42 | 7.21 | 0.02 |
| 15 | 3352.28 | 28.623 | 0.114 | 3356.14 | 3336.85 | 10.42 | 0.014 |
| 16 | 3865.35 | 89.65 | 0.668 | 3874.99 | 3855.7 | 0.889 | 0.038 |

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Fig 6: FT/IR analysis of the organic extract of uninoculated MSPM.

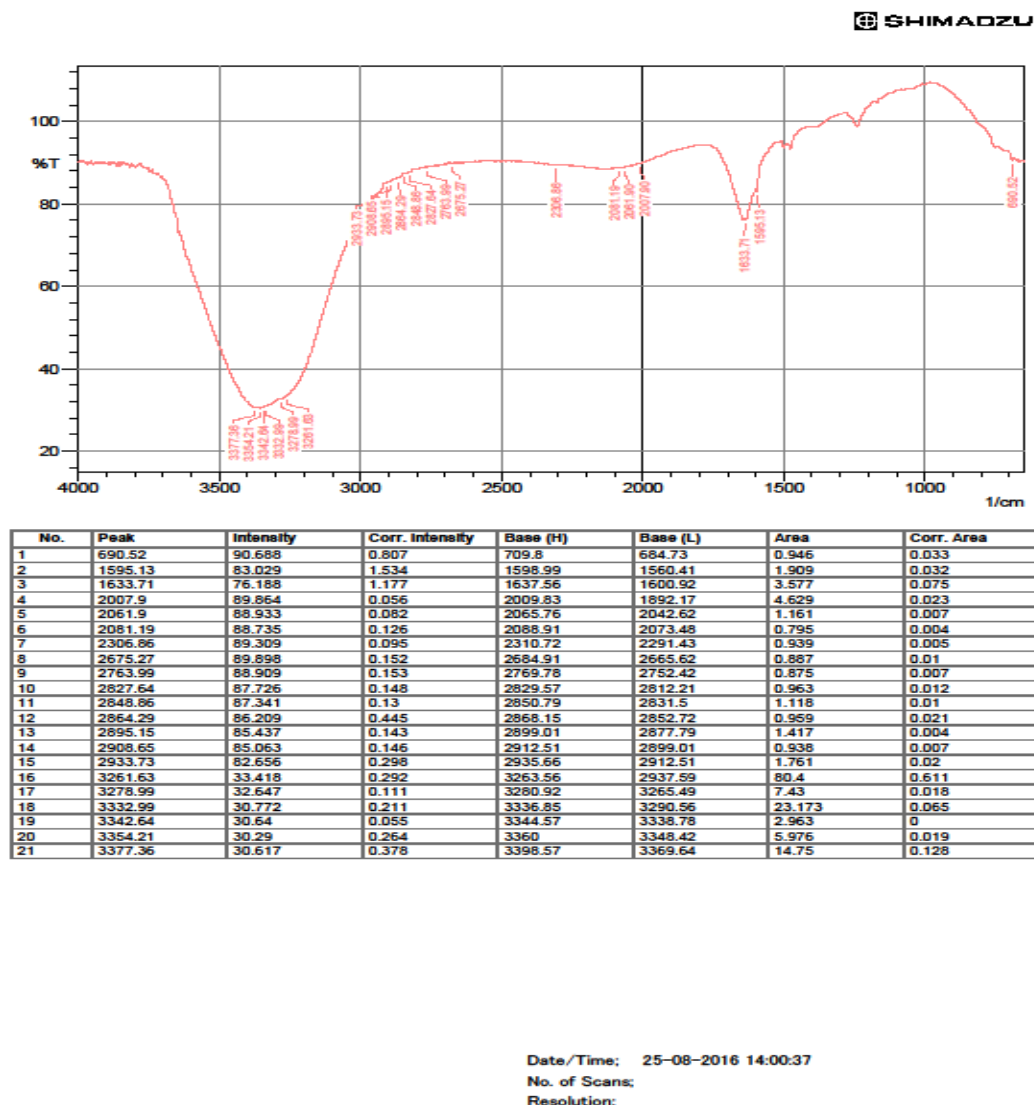


Fig 7: FT/IR analysis of the organic extract of MSPM inoculated with *Bacillus subtilis* sp3 and incubated for 24 hours.

3.6. Optimization of pH and incubation period for maximum growth

The selected organism, *Bacillus subtilis* sp3 was capable of degrading phenol and accumulating PHB progressively along the logarithmic phase of growth. Hence an attempt was made to optimize the conditions, particularly pH (Fig.8) and incubation period (Fig.9) for the maximum growth of the organism. The pH optimization studies were conducted at different pH from 5 to 10. A maximum cell concentration could be observed at pH 7. The biomass concentration at pH 5 and 6 were less. The cell concentration was found to be decreasing progressively in the alkaline range also. On evaluating the effect of incubation period on biomass, 22 o 26 hour was found to be optimum for maximum cell concentration.

Hence a pH of 7 and incubation period of 24 hour were selected as the optimum conditions for phenol biodegradation.

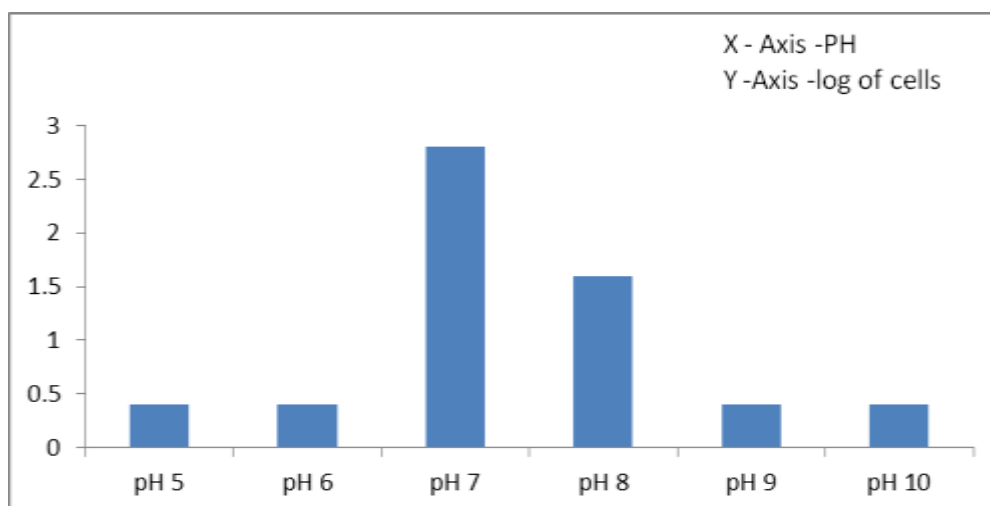


Fig 8: Effect of pH on growth of *Bacillus subtilis* sp3 in MSPM.

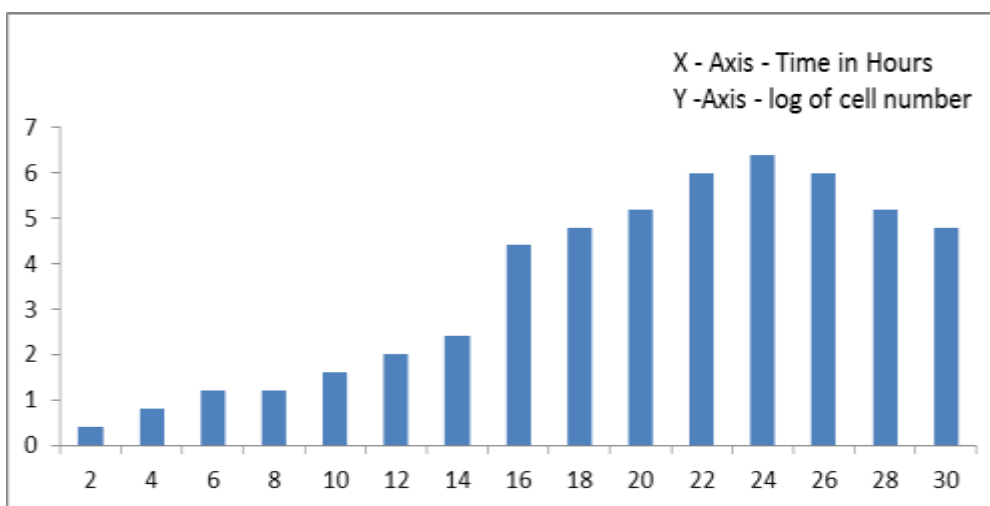


Fig 9: Effect of incubation time on the growth of *Bacillus subtilis* sp3 in MSPM.

3.7. Treatment of waste water from paper pulp industry: Phenol concentration of the waste water collected from paper industry were decreased after the bacterial treatment. Correspondingly there was progressive decrease in Chemical oxygen demand on treatment with the selected isolate for 24 hours (Table 2).

Table 2: Treatment of the phenolic effluent with the selected isolate *Bacillus subtilis* sp3.

| Parameters | Before treatment | After treatment |
|----------------------------|------------------|-----------------|
| Phenol concentration(mM) | 9±0.5 | 5±0.3 |
| COD of waste water (mg/ml) | 2380±5 | 856.8± 4 |

Maximum percentage reduction of COD was 64% after 24 hours of treatment. This is effected because of the reduction in the concentration of phenol along with it reduction in the load of other organic contaminants. Colour, pH variation and high COD are the main problems associated with paper mill effluents. Various derivatives of phenol like dichlorophenol and pentachlorophenol are also observed in general.^[13] In the present study the bacteria showed active growth in the diluted waste water in lab conditions and could achieve considerable COD reduction. In the aerobic treatment systems of paper and pulp effluents bacteria show better performance, mainly due to the ability to tolerate wide pH ranges. Further studies are being progressed to optimize the degradation process.

Treatment systems like titanium oxide oxidation and photo fenton reactions are expensive as they are high energy utilizing. So biological treatment systems are preferred to physico chemical systems in phenolic waste treatment.

We have also reported earlier about *Alcaligenes* sp d₂ accumulating PHB upon phenol stress where the accumulation started nearing to complete utilization of phenol.^[2] In many of the cases the property of phenol degradation by bacterial species have been linked to stress management mechanisms within the species.^[14] Polyhydroxialkanoates are reported to be produced in considerable amounts through acidogenic fermentation where effluents from paper mill are used. Optimization of production parameters are required according to the constituents of the effluent.^[15] This is a feature to be exploited for the production of biodegradable plastics using inexpensive sources.

CONCLUSION

Phenol is a toxic organic compound which is usually found associated with many industrial effluents. Its biodegradation is often challenged by the fact that phenol itself is antibacterial in its action. However many attempts are being made to isolate phenol degrading bacterial strains from soil in the view point that bioremediation strategies could be developed and the molecular biology of the same may be explored. In the present study the selected organism could degrade phenol within a short span of time. The organism was characterized as *Bacillus subtilis* sp3 which showed accumulation of PHB along with phenol degradation. Further investigations into the process can bring out fruitful strategies for optimization and scaling up of bioremediation of phenolic effluents and PHB production.

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