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Degradation of Phenol by Selected Strains of Bacillus Species Isolated from Marine Water

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ABSTRACT

Several external factors can limit the rate of biodegradation of organic compounds. These factors may include temperature, pH, oxygen content and availability, substrate concentration and physical properties of contaminants. Each of these factors should be optimized for the selected organism for the maximum degradation of the organic compound of choice. The optimization of the substrate concentration in phenol biodegradation is particularly important since it inhibits the growth of the organism at higher concentrations. Thus the present study was planned to provide scientific justification for the biodegradation of phenol by *Bacillus* sp., which were isolated from marine water. Marine water samples were collected and processed for the isolation and identification of phenol degrading bacterium. Based on the morphological, physiological and biochemical characteristics the isolates were identified as two different species of *Bacillus*. Basal minimal medium was prepared and inoculated with these two different species of *Bacillus* for the study of biodegradation of phenol. Biomass study was carried out to measure the cell growth of *Bacillus* sp., against phenol. At different time intervals of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs the turbidity of the culture was measured and observed for biomass study at 600 nm. After 120 hrs the highest biomass was observed in both strains which were indicated by increased cell growth. Cell free supernatant was collected and degradation of phenol was measured by rapid colorimetric method using 4-amino antipyrine. Optimization of various parameters was carried out to analyse the effect of various temperatures, pH, Nacl concentrations, urea concentrations, phenol concentrations, glucose concentrations on phenol degradation. After the appropriate time period of degradation, the concentration of phenol in the culture medium was measured by the technique of Thin Layer Chromatography using the Rf values of samples compared with Rf values of standard phenol solution. Rf values were found to be 0.8 and 1.0 for SI and SII isolates. Therefore, the result revealed that the *Bacillus* strains isolated from marine water have the ability to degrade phenol which is one of the hazardous pollutants of environment and these strains could be used as an effective biodegrading agents of phenol which degrade phenol in to non-toxic by-products.

Key words : Phenol, biomass, degradation, marine, *Bacillus*, 4-amino antipyrine, minimal medium.

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INTRODUCTION

Environmental pollution due to anthropogenic activity has spread to all types of ecosystems. Marine and fresh water, soils and air have been impacted by the dispersion of contaminants (Margesin and Schinner)[13]. Waste water released from various industries has become a major concern for environmentalists. Industrial effluents contain various toxic metals, harmful gases and several organic and inorganic compounds. Both the quality and quantity of effluent result in various impacts on the availability of good quality water as well as on marine environment (Wahaab).[23]

Phenols are aromatic compounds that are characteristic pollutants in waste water and effluents from chemicals, petrochemicals, pharmaceuticals, textiles and steel industries. The unwholesome and environmentally unacceptable pollution effects of the phenolic effluent have been reported world wide. Phenol contaminants are relatively soluble in water and accumulate in soil, resulting in extensive surface water, ground water, and soil contamination owing to its severe toxicity. As water is precious commodity (Baroniya *et al*, Murhekar,) [3,4] according to the standard set by United State Environmental Protection Agency (USEPA) surface water must contain less than 1 microgram/liter phenol (Nor *et al*).[16]

Several industrial processes, such as pesticide, chemical and pharmaceutical production as well as gas and oil extraction, generate thousands of millions of litres of saline to highly saline wastewaters (Lefebvre and Molett).[12]

Phenol is also designated as Carbolic acid, Hydroxybenzene, Phenic acid, Phenic monohyroxybenzene, Phenylic acid, Phenyl hydroxide, Oxybenzene, Benzynol, Monophenol, Phenyl hydrate, phenylic alcohol, Barker's P and S, Phenol alcohol. Acute exposure of phenol causes central nervous system disorders. In recent years, a great deal of research work has been directed toward the development processes in which enzymes are used to remove phenolic contaminants (Ghioureliotis and Nicell).[7] Vapours and liquids are toxic and easily absorbed through the skin. Once inhaled, vapours corrode the respiratory tract and the lungs. Severe burns result from the liquid coming into contact with skin and eyes (phenol is a powerful protoplasmic poison). Long-term exposure paralyzes the central nervous system and damages the kidneys and the lungs. Paralysis may cause death.

The biological degradability of natural phenols is generally very good with the result that there is scarcely any accumulation in plants or animals. Aerobic bacterial degradation involves complete breakdown to carbon dioxide. Degradation of phenol occurs as a result of the activity of a large

number of microorganisms including bacteria, fungi and actinomycetes. Bacterial species include *Bacillus sp*, *Pseudomonas sp*, *Acinetobacte*r *sp*, *Achromobacte*r *sp* etc. *Fusarium sp*, *Phanerocheate chrysosporium*, C*orious versicolo*r, *Ralstonia sp*, *Streptomyces sp* etc are also proved to be efficient fungal groups in phenol biodegradation. However, these microorganisms suffer from substrate inhibition at higher concentration of phenol, by which the growth is inhibited (Prieto *et al*).[17]

Most of the efficient phenol degrading microorganisms are capable of using phenol as the sole source of carbon and energy for their growth and metabolism. Microorganisms capable of degrading phenol do so with the action of variety of enzymes. The enzyme catechol 2, 3 dioxygenase cuts the benzene ring at the ortho and meta cleavage are finally consumed by the microbes with the help of various enzymes through the TCA cycle resulting CO_2, metabolites and energy (Dabadatta and Rajdeep).[6]

MATERIALS AND METHODS

Five sea water samples were collected from following sites in Chennai and Nagapattinam District. Kovalam beach, Kovalam, Chennai. Nagoor beach, Nagoor, Nagapattinam District. Besant nagar beach, Chennai. Thiruvanmiyur beach, Chennai. Shollinganallur beach, Chennai. Samples were collected in sterile wide mouthed screw capped bottle. The samples collected in sterile container were transferred to the laboratory for processing. The samples were serially diluted and plated on sterile Nutrient agar plates which contain 0.1g of phenol/100ml media, by spread plate technique. The plates were incubated at 34^{o}C for 24-48 hours. After incubation period the plates were observed. Pure cultures of all morphologically suspected colonies were maintained in slants and stored for further characterization. (Ravikumar *et al*).[18] The pure cultures obtained were further characterized for the identification of phenol degrading *Bacillus* species.

Determination of Biodegradation of Phenol (Awan, 2013)

Biodegradation of phenol was carried out by using shake flask method. A loopful of pure *Bacillus* strain was inoculated into 100 ml basal minimal medium, with the addition of 100 mg of phenol in 250 ml Erlenmeyer flask. This flask was incubated at room temperature with continuous shaking at 150 rpm in thermostat incubator for 24-48 hours. At different time intervals samples were centrifuged at 3000 rpm for 20 minutes and analyzed by rapid colorimetric method.

Biomass Study (Debadatta and Rajdeep, 2012)

1 ml culture was taken in a centrifuge tube and allowed to centrifuge at 10,000 rpm for 5 minutes. The supernatant is used for analysis of phenol degradation by rapid colorimetric method. The pellet was washed with distilled water several times and the optical density of bacterial suspension was determined at 600 nm by taking distilled water as blank.

Analytical Method (Rapid Colorimetric Method)

To 50 ml of aliquots, 0.3 ml of 2% aqueous 4-amino antipyrine solution and 1 ml of 2N NH_4OH were added. After mixing the content thoroughly 1 ml of 2 % K_3FeCN_6 is added. Absorbance of red colour produced is measured at 510 nm in colorimeter.

OPTIMIZATION OF VARIOUS PARAMETERS FOR DEGRADATION OF PHENOL

Effect of different pH

A sequence of experiments for degradation of phenol was conducted to reveal effect of pH at 35^oC at different time in shaking incubator at 120 rpm. pH maintained 5 to 9 in culture of basal minimal medium. Samples were collected and analyzed calorimetrically for phenol concentration.

Effect of different Temperatures

A series of experiments for degradation of phenol was conducted to reveal effect various temperatures on the degradation of phenol at neutral pH in shaking incubator at 120 rpm. Temperatures were maintained at 29 to 37^oC. Samples were collected and analyzed colorimetrically for phenol concentration.

Effect of different Glucose concentrations

A series of experiments were performed to know the effect of glucose on the degradation of phenol at 35^oC and at pH 7. Glucose concentration maintained 0.1 to 0.5% in culture of basal minimal medium. Samples were collected and analyzed colorimetrically for phenol concentration.

Effect of Different Sodium Chloride concentrations

The sequences of experiments were performed to know the effect of Nacl on the degradation of phenol at 35^oC and at pH 7. Nacl concentrations were maintained 1 to 5% in culture of basal minimal medium. Samples were collected and analyzed colorimetrically for phenol concentration.

Effect of different Phenol concentrations

A series of experiments for degradation of phenol was conducted to reveal degradation of various phenol concentrations at neutral pH in shaking incubator at 120 rpm. Phenol concentrations were maintained 100 to 500 mg. Samples were collected and analyzed

colorimetrically for phenol concentration.

Effect of different Urea concentrations

A series of experiments for degradation of phenol was performed to know the effect of urea on degradation of phenol at neutral pH in shaking incubator at 120 rpm. Urea concentrations were maintained 0.1 to 0.5%. Samples were collected and analyzed colorimetrically for phenol concentration.

Thin Layer Chromatography

The aliquots were spotted on a TLC plate. The solvent system was acetic acid, distilled water in 1:99 ratio. The concentrations of phenolic compound was measured based on the total integrated area of the designated spot compared with control (Rf value of 0.57).

RESULTS AND DISCUSSION

About five marine water samples were collected in sterile container from the following sites and were transferred to the laboratory for processing. Growth was observed in all samples. More than 200 CFU/ml was observed in sample-1, 2 and 5. More than 230 CFU/ml was observed in sample-4. More than 250 CFU/ml was observed in sample-3. Two different colonies which are larger in size and high in number were used for further analysis and these two types of colonies were named as S-I (Strain-I) and S-II (Strain-II). The colony morphology of S-I and S-II strains were Large, irregular, flat colonies and Opaque, wrinkled, irregular colonies respectively which were identified as *Bacillus* sp.

At different time intervals of 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs the turbidity of the culture was measured and observed for biomass study at 600 nm. After 120 hrs the highest biomass was observed in both strains which were indicated by increased cell growth (Table. 1).

Table. 1 Biomass Study of Strains

Hours of Incubation (hrs)	OD value at 600 nm	
	S-I	S-II
24	0.8	0.5
48	1.2	0.9
72	1.5	1.3
96	1.7	1.6
120	1.9	1.8

Analytical Method (Rapid Colorimetric Method)

After addition of 4-amino antipyrine, NaOH and K_3FeCN_6 with the supernatant of culture, dark red colour was produced in aliquots of the strains. The absorbance of red colour produced was measured at 510 nm in colorimeter.

Optimization of various parameters for degradation of phenol

Effect of different pH

At various time intervals the OD value of the aliquots were measured at 510 nm after the addition of 4-amino antipyrine, NaOH and K_3FeCN_6. The degradation of phenol was increased with the increase in pH (Figure. 1a and b).

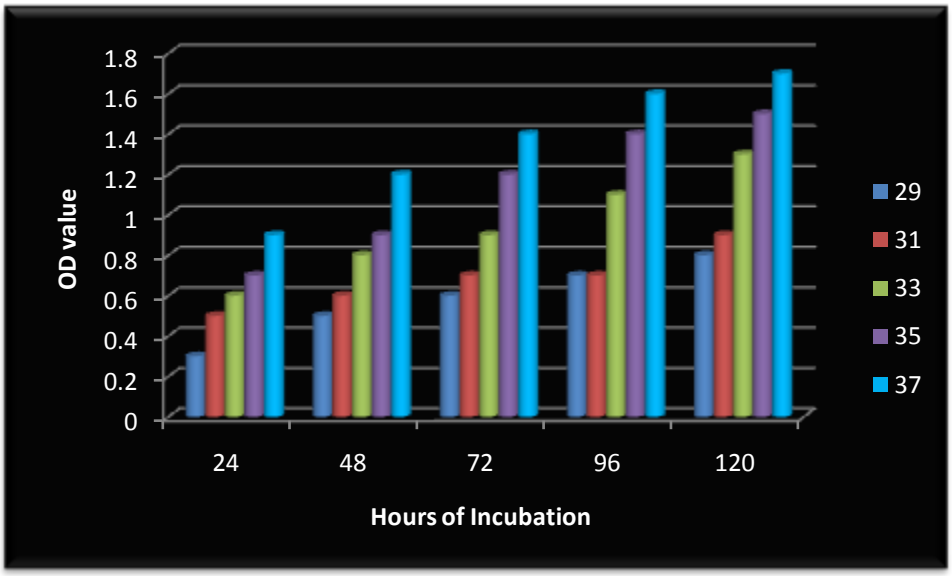

Figure.1a: Effect of Different pH on Phenol degradation by S-I strain

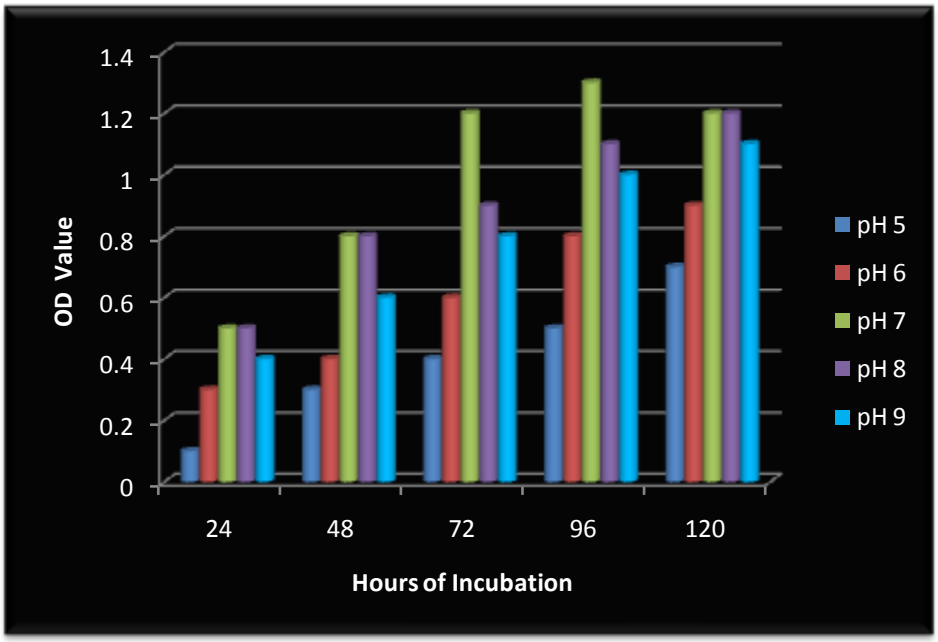

Figure. 1b: Effect of Different pH on Phenol degradation by S-II strain

Effect of Different Temperatures

The optimum temperature for phenol degradation was tested by the series of experiments with various temperatures. The temperature range between 35°C as well as 37°C was considered as optimum for effective phenol degradation (Figure. 2a and b).

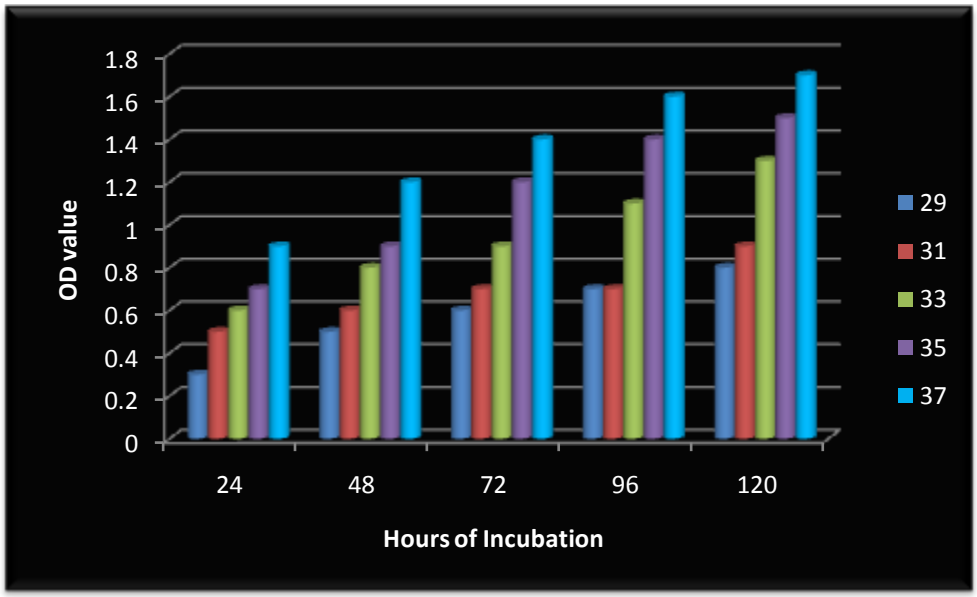

Figure. 2a:Effect of Different Temperatures on Phenol Degradation by S-I strain

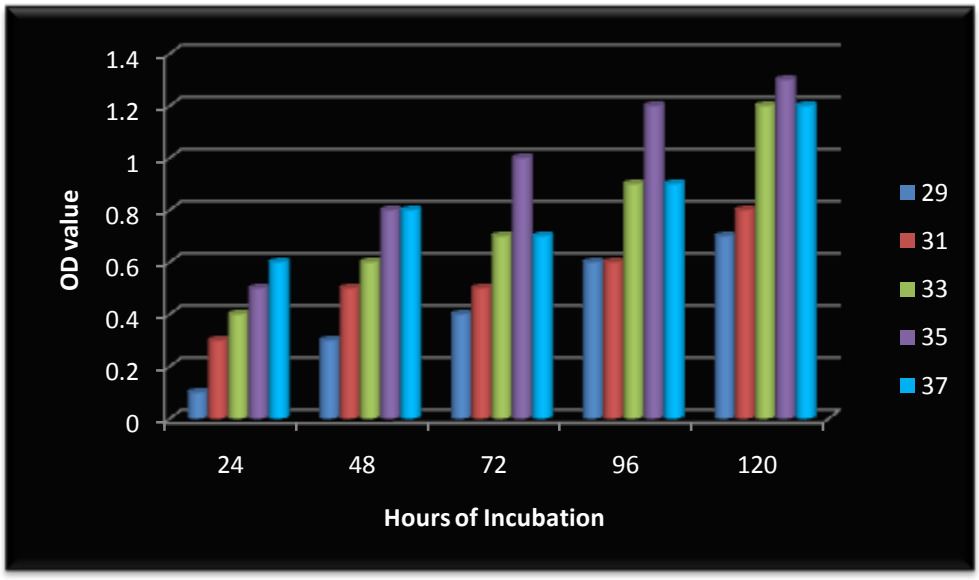

Figure. 2b: Effect of Different Temperatures on Phenol Degradation by S-II strain

Effect of Different Glucose Concentrations

The degradation rate decreased with the increased concentration of glucose. The optimum concentration of glucose for the phenol degradation was 0.3% for the two strains of *Bacillus* (S-I and S-II) (Figure. 3a and b).

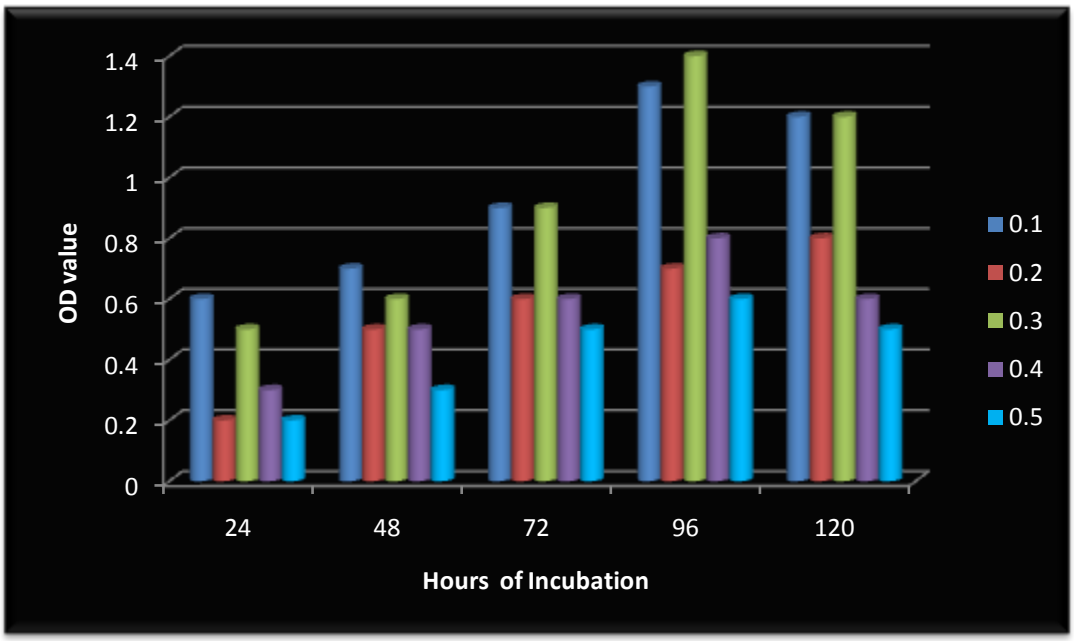

Figure. 3a :Effect of Different Glucose Concentrations on Phenol Degradation by S-I strain

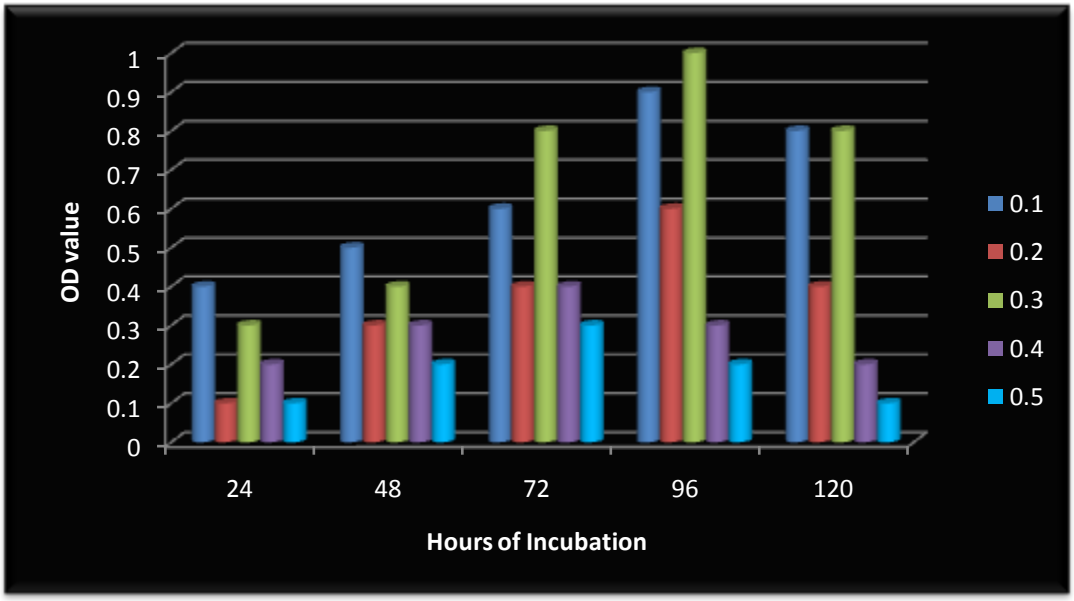

Figure. 3b: Effect of Different Glucose Concentrations on Phenol Degradation by S-II Strain

Effect of Different Sodium Chloride Concentration

The degradation capacity of strains decreased with the increasing Nacl concentrations which leads to decrease growth rate and denaturing the proteins. 2% of Nacl was considered as optimum for the phenol degradation effectively (Figure. 4a and b).

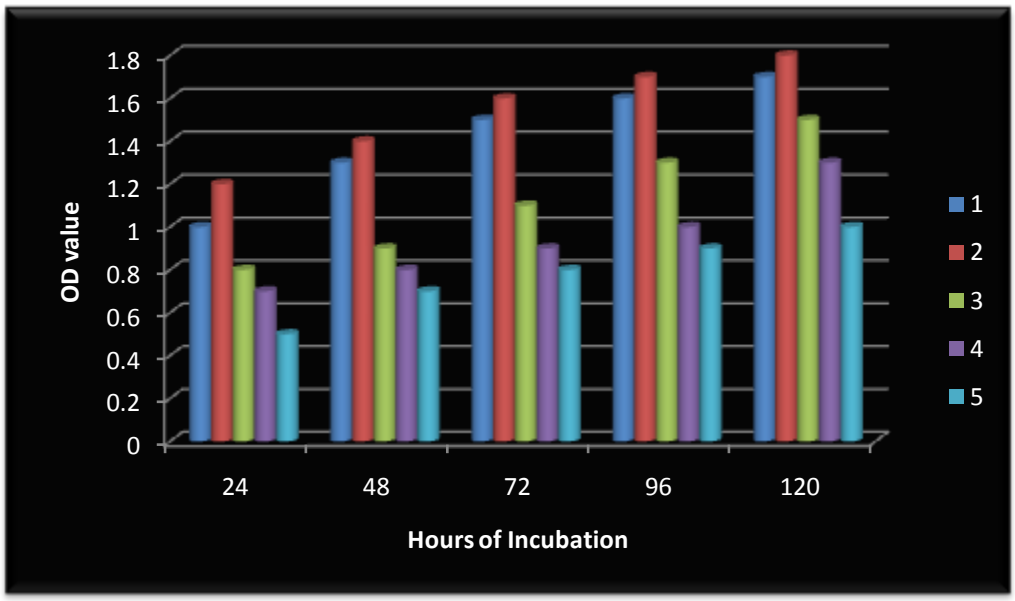

Figure. 4a: Effect of Different Nacl Concentrations on Phenol Degradation by S-I Strain

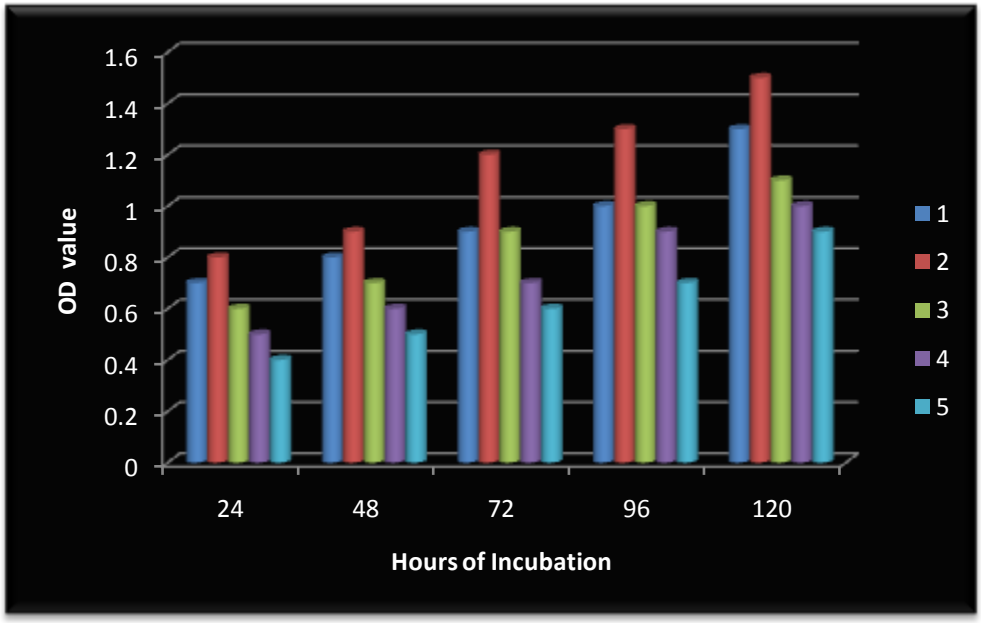

Figure. 4b:Effect of Different Nacl Concentrations on Phenol Degradation by S-II Strain

Effect of Different Phenol Concentrations

Increased phenol concentration leads to increasing of time to degrade phenol by microorganisms. The increased concentration (500 mg) of phenol in culture was degraded with in 120 hrs of incubation (Table. 8 and Figure 5a and b).

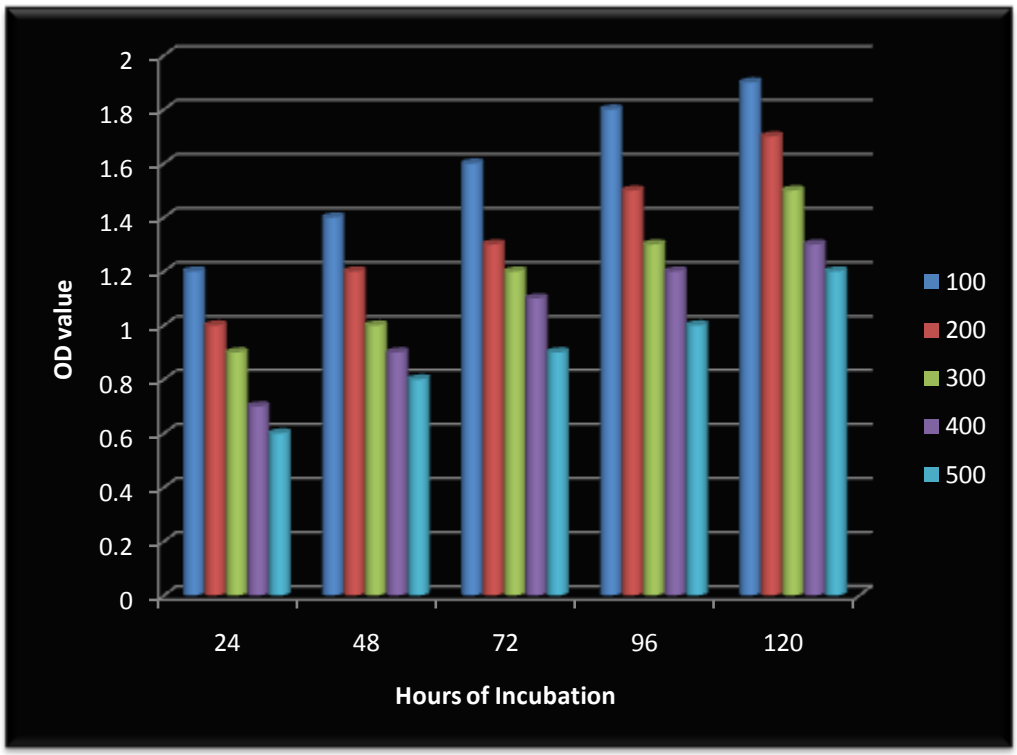

Figure. 5a :Effect of Different Phenol Concentrations at Neutral pH by S-I Strain

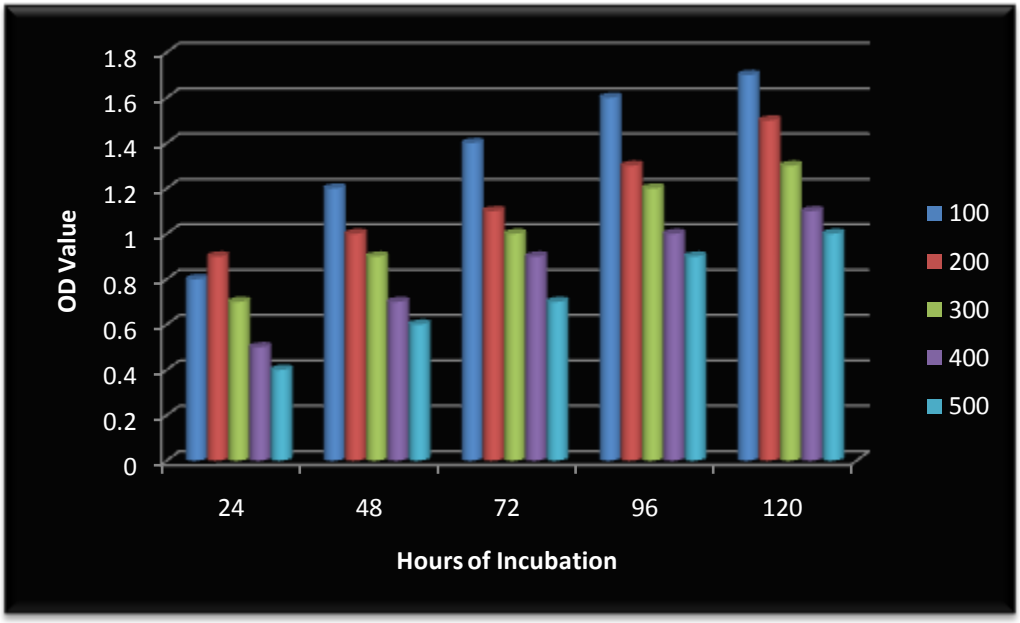

Figure. 5b:Effect of Different Phenol Concentrations at Neutral pH on S-II Strain

Effect of Different Urea Concentrations

Urea acts as nitrogen source for the growth of microbes, but increased concentration of urea decreased the degradation of phenol. The optimum concentration of urea was 0.2% for the S-I and S-II *Bacillus* strains (Figure. 6a and b).

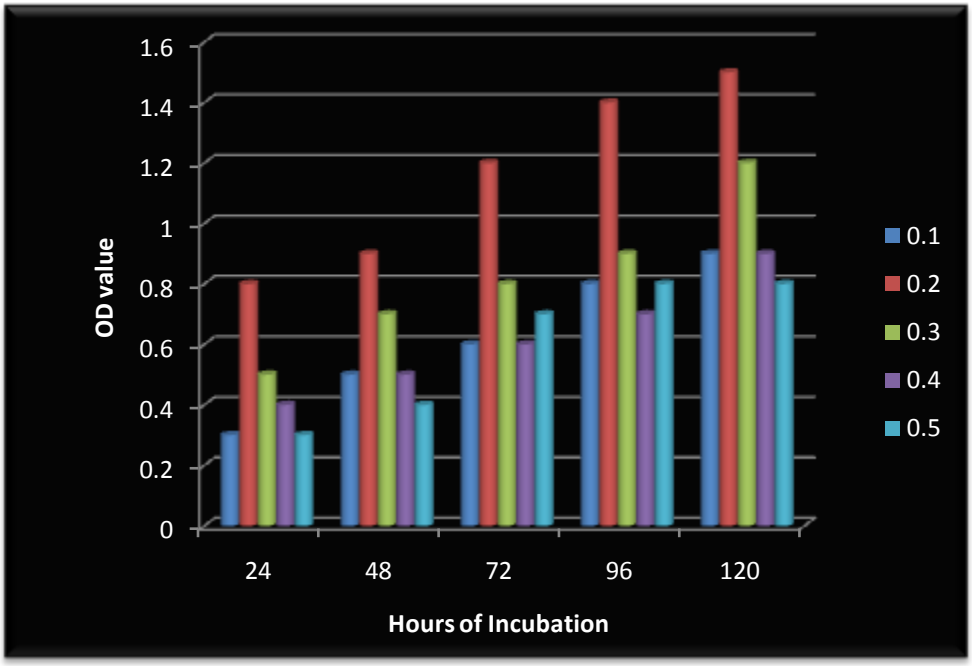

Figure. 6a: Effect of Different Urea Concentrations on Phenol Degradation by S-I Strain

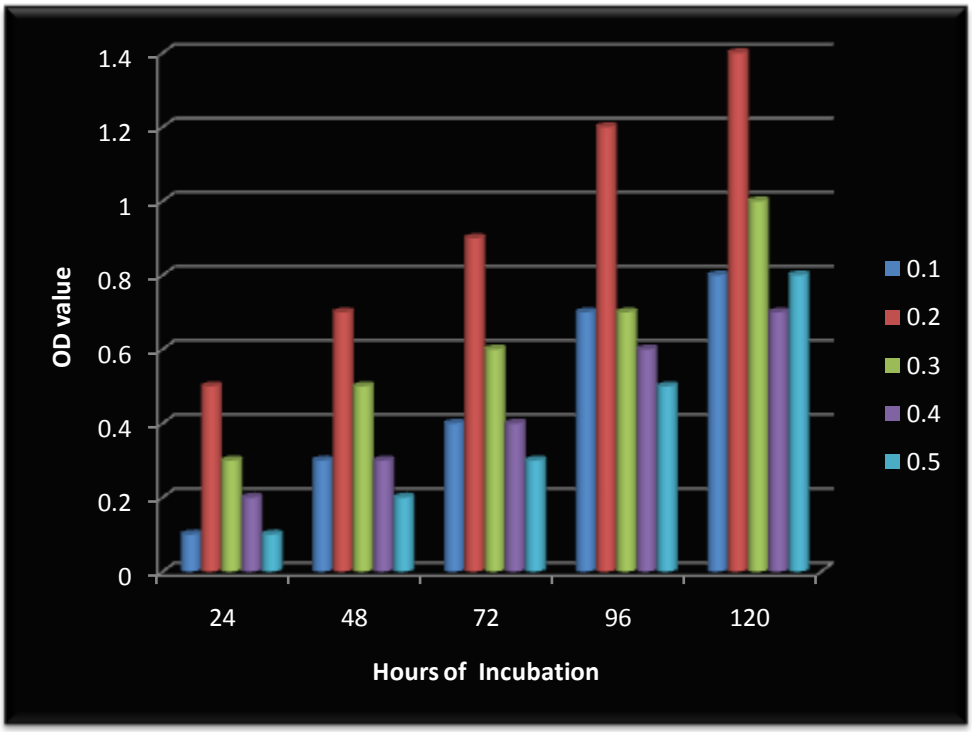

Figure. 6b:Effect of Different Urea Concentrations on Phenol Degradation by S-II Strain

Thin Layer Chromatography

Thin Layer Chromatography was carried out to determine the phenol degradation by both the isolates. The distance travelled by solute and solvent was measured and the Rf values were calculated by following formula. Rf values were found to be 0.8 and 1.0 for SI and SII isolates.

Biodegradation of phenol and associated phenol containing compounds by way of various microorganisms is to be the focus of scientific concentration since many years. A huge number of innate and artificial organic compounds are degraded by microorganisms as tiny proportion of their ordinary metabolism for energy and development.

The bio treatment of industrial hypersaline wastewater streams is problematic because conventional wastewater treatment is strongly inhibited by high salt concentrations and additionally so by phenol itself. However, relatively little is known about the distribution and phenol biodegradation potential of halophilic bacteria. In this regard, the identification of halophiles capable of phenol degradation was an essential component in the evaluation and development of efficient bio treatment options for phenol containing hyper saline wastewaters (Bonfa *et al*.) [4]

In this study two different species of *Bacillus* isolated from marine water in different areas, were shown to degrade and grow on 100 mg phenol as the sole carbon source in media. The isolates were identified as *Bacillus* sp., which were degrading phenol up to 500 mg.

There are factors that can control degradation ability or metabolism of microorganisms by either preventing or stimulating growth of the organisms and more carefully by affecting gene expression. Some of the factors that affect the biodegradation of phenols as well as other xenobiotic compounds are chemical structure and compound toxicity. Other is the environmental factors such as pH, temperature, nutrient (concentration of Nacl, urea, glucose, phenol) Solomon *et al*. [21]

In the present study isolated bacteria have degraded the phenol in the pH of 7. Maximum of reduction was recorded at pH 7 by these (S-I and S-II) strains. Similar results were given in the work of Alexander and Robertson.[1] They experimented that the majority of organisms could not situate for pH range below from 5.0 or above from 9.0. At high or low pH values acid or base could burst during in to cells further simply, because they have affinity to survive in undissociated structure underneath these circumstances and electrostatic force cannot shun them from incoming cells and the optimal conditions for phenol removal were found to be pH of 7 by the native bacterial strains of waste water.

Each life form has a minimum, optimum and maximum temperature for growth. In our study, bacteria isolated from marine water were checked for their degradative ability and it was found that 37^{o}C was the optimum temperature for the degradation of phenol. Maximum percentage of reduction was recorded at 37^{o}C by these two strains. As well as Chitra [5] and Awan *et al* [2] described that while the temperature improved from series of 30^{o}C there was due to cell worsen,

no phenol degradation was observed. So the phenol degradation is a temperature dependent procedure. The increase tariff usually reaches optimum in the temperature of range between 35^{o}C as well as 37^{o}C.

The present study reveals that, the selected strains showed maximum degradation of phenol with the addition of 0.2% glucose. The degradation rate decreased with the increased concentration of glucose. This might be due to the catabolic respiration by glucose; ie., the presence of glucose could inhibit the utilization of target substances. This is correlated with the work of Kar *et al* [9] Satsangee and Ghosh[19] which showed the effect of glucose on phenol degradation and the results indicated that when a mixed substrate (phenol and glucose) was used, phenol acclimatized population showed initial preference for phenol to glucose concentration. A glucose concentration of 0.5% repressed the induction of phenol oxidation through glucose did not fully repress utilization of phenol. The phenol removal efficiency was determined by the addition of different glucose concentrations at neutral pH.

In present study, the isolated bacteria were investigated for their biodegradation ability to degrade phenol and found that 2% Nacl was the optimum concentration for the degradation of phenol in these two strains of *Bacillus*. The similar results were given by Veenagayathri and Vasudevan [22] and Ravikumar *et al* [18] that is the degradation capacity of the selected strains decreased with the increasing Nacl concentration, which leads to decrease the growth rate and denaturing the proteins. Degradation was not occurred at high Nacl concentration. This might be due to the higher salt concentration which reduces the microbial activity. Biodegradation of phenol in the presence of high concentration of Nacl has been already reported that it leads to decreased growth rate and denaturing the proteins.

In this study, the isolated strains degraded phenol up to 500 mg of concentration within 120 hrs, at neutral pH and 37^{o}C of optimum temperature. As well as Kargi and Eker [10] have been shown several reports that microbial growth and concomitant biodegradation of phenol are hindered by the toxicity exerted by high concentration of substrate itself. A high concentration of phenol is usually inhibitory to the growth of microorganisms. Ursin [21] and Hwang *et al* [8] have described the concentration of phenol seems to play a crucial role of degradation. In our study, the maximum removal of phenol by selected strains of *Bacillus* was observed at 0.2% of urea, which used as nitrogen source and it degrade the phenol at neutral pH with in 120 hrs. It similar to the analysis of Naresh *et al*[15] stated that *Staphylococcus aureus* has potential to remove maximum phenol at 0.2% concentration of urea and ammonium chloride and the maximum removal of phenol was observed at 0.2% of ammonium chloride.

In present study, the solvent system was acetic acid, distilled water in 1:99 ratios and the concentration of phenol was measured based on the Rf values of spots compared with control. The lower Rf value of S-I indicates the higher utilization of phenol compared with S-II in 120 hrs of incubation. As well as Lin *et a*[10] reported that the concentration of phenolic compounds based on the total integrated area of the designated spot compared with control on TLC plate.

Additional studies on the application of halophiles for the treatment of industrial waste waters should provide the necessary knowledge base for the development of robust waste water treatment systems that can become an accepted and valuable treatment option for hypersaline industrial waste streams containing phenol. In future technologies, microbial systems might be the potential tools to deal with the environmental pollutants.

The degradation of pollutants at high salt concentrations is still extremely limited. This may simply be due to the fact that the potential of halophilic bacteria for biodegradation of toxic compounds has never been systematically investigated. Our lack of knowledge by no means implies that halophilic bacteria do not have a potential to degrade toxic compounds. The opposite may be true- a world of unusual microorganisms is waiting to be tested for useful properties.

The present result suggested that the bacterial strains isolated from sea water are capable of degrading phenol effectively. However, further evaluation is required for the definite conclusions contributing to the phenol degrading capacity of strains. These strains could be acts as agents to reduce the phenol pollution which is more toxic to our environment and it is the less expensive method of bioremediation. This study can also focus on more cost effective applications of native bacterial strains for phenol degradation at large scale in industries, where it pose an alarming problem due to its detrimental health effects on different organisms and human beings.

CONCLUSION

From this research report, it is concluded that the *Bacillus* strains of marine water could be used as a good biodegrading agent of phenol which cause pollution on environment especially in marine ecosystems. These strains degrade phenol effectively by producing non-toxic by-products, which cannot cause any side effects or harmful effects to living beings.

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