Biodegradation Study of Phenol by *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 Isolated from Contaminated Soil

Thesis submitted in partial fulfillment
for the award of the degree

Of

Master of Technology (Research)
In
Chemical Engineering

By

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Under the Supervision of

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Dedicated to

My parents

Ashadevi Shamrao Patil

&

Shamrao Maruti Patil

CERTIFICATE

This is to certified that the thesis entitled "**Biodegradation Study of Phenol by *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 Isolated from Contaminated Soil"** submitted by **Sangram Shamrao Patil (611CH107)** at National Institute of Technology, Rourkela is a record of bonafide research work under my supervision and is worthy of consideration for the award of the Degree of Master of Technology (Research) in Chemical Engineering of the institute. The candidate has fulfilled all prescribed requirements and the thesis, which is based on candidate's own work, has not been submitted elsewhere for a degree or diploma.

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SANGRAM SHAMRAO PATIL

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ABBREVIATIONS

EPA	Environmental Protection Agency
NaOH	Sodium Hydroxide
NIST	National Institute of standards and technology
IS	Indian Standards
WHO	World Health Organization
ATSDR	Agency for Toxic Substances and Disease Registry
TiO_2	Titanium dioxide
GAC	Granular activated carbon
H_2O_2	Hydrogen Peroxide
DIPE	Diisopropyl ether
MTCC	Microbial Type Culture Collection
mM	Millimole
µmol	Micromole
NADH	Nicotinamide Adenine Dinucleotide
NaCl	Sodium Chloride
2-HMSA	2-Hydroxymuconic Semialdehyde
RSM	Response Surface Methodology
CCD	Central Composite Design
BBD	Box - Behnken Design
ATCC	American Type Culture Collection
rpm	Rotations Per Minute
vvm	Volume per Volume per Minute
NCIM	National Collection of Industrial Microorganisms
Cu	Copper
Mn	Manganese
mg/l	Milligram per liter
v/v	Volume per Volume
w/v	Weight per volume
UV	Ultra Violet

rDNA	Ribosomal DNA
MRVP	Methyl red- Vogues Proskauer
SEM	Scanning Electron Microscope
O.D.	Optical Density
TAE	Tris-acetate-EDTA
PCR	Polymerase Chain Reaction
µl	Micro liter
BLAST	Basic local Alignment Search tool
NCBI	National Centre for Biotechnology Information
MEGA	Molecular Evolutionary Genetics Analysis
APHA	American Public Health Association
BDT	BigDye® Terminator
RDP	Ribosomal Database Project
OFAT	One Factor at a Time
EDTA	Ethylene Diamine Tetra acetic Acid
CTAB	Cetyltrimethylammonium bromide
nr database	non-redundant database

NOMENCLATURE

μ:	Specific growth rate, 1/h
μ_{max}:	Maximum specific growth rate, 1/h
K_s:	Half-saturation coefficient, mg/l
K_i/K_I:	Substrate inhibition constant, mg/l
q:	Degradation rate, 1/h
q_{max}:	Maximum degradation rate, 1/h
S_0:	Initial substrate concentration, mg/l
k :	Constant, mg/l
S_m :	Critical inhibitor concentration, mg/l
n, m:	Empirical constants

ABSTRACT

Water pollution by phenols is a major environmental problem in present days. Phenol is a highly hazardous and toxic substance emitted to the environment by the effluent from various industries. Environmental Protection Agency has set the limits for concentration of phenol in wastewater discharge are 0.5 mg/l for surface waters and 1 mg/l for the sewerage system Therefore, industrial effluents containing phenol require proper treatment before being discharged into the environment. There are various methods available for removal of phenol from wastewater. Among these, Biological treatment of phenolic effluent is attractive than that of other alternatives as it is cost effective and produces non toxic end products. Biodegradation of phenol mainly depends on the efficiency of the microbe, concentration of media components and the physiological conditions.

In the present study two different phenol contaminated soils (one with effluent from paper mill and the other with crude oil) has been chosen to isolate highly efficient microbes. Aerobic bacterial strains PS3 and OS1 have been isolated from the soil contaminated with paper mill effluent and crude oil respectively. Strain PS3 has been found to tolerate 1500 mg/l of phenol, while the strain OS1 tolerate up to 1250 mg/l of phenol. On the basis of morphological, biochemical and molecular characteristics, strain PS3 and strain OS1 have been identified as *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 respectively.

Optimization studies on growth and degradation has been carried out by using Plackett-Burman Design and central composite design (CCD) to evaluate optimum values of medium components and physiological conditions. Most significant factors have been screened using Plackett-Burman design from nine important variables. Temperature, pH, phenol concentration and inoculum size have been found significant for *Burkholderia* sp. PS3 while pH, temperature, phenol concentration, inoculum size and $(NH_4)_2SO_4$ concentration have been found significant for *Bacillus pumilus* OS1. These factors have been optimized by central composite design with correlation coefficient of 0.9679 and 0.9827 for strain PS3 and OS1 respectively. For *Burkholderia* sp. PS3, maximum phenol degradation of 99.96% has been predicted at pH - 7.18, temperature - 28.9°C, phenol - 297.9 mg/l and inoculum size - 5.04% (v/v). A maximum phenol degradation of 99.99% has been predicted for *Bacillus pumilus* OS1 at pH - 7.07, temperature - 29.3°C, phenol - 227.4 mg/l, inoculum size - 6.3% (v/v) and $(NH_4)_2SO_4$ - 392.1 mg/l. The predicted

optimum degradations have been validated by experiments and the experimental degradation has been found to be 99.88% and 99.90% for *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 respectively.

Haldane model has been found to fit the experimental data and the growth kinetic parameters; maximum specific growth rate, half-saturation coefficient and the substrate inhibition constant have been found to be μ_{max} = 0.0436 h^{-1}, K_s = 29.43 mg/l and K_i = 839.90 mg/l for *Burkholderia* sp. PS3, and μ_{max} = 0.0370 h^{-1}, K_s = 38.27 mg/l and K_i = 587.62 mg/l for *Bacillus pumilus* OS1.

To characterize the enhancement in tolerance and phenol degradation potential, the isolated strains have been immobilized to calcium alginate beads. The immobilized cells of *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 has been found to degrade 31.27% of 1500 mg/l and 46.88% of 1250 mg/l in comparison to 17.3% and 28.32% for respective free cells under the same condition. The tolerance of immobilized *Burkholderia* sp. PS3 has been increased to 1600 mg/l of phenol, while immobilized *Bacillus pumilus* OS1 has been found to tolerate up to 1350 mg/l phenol.

Keywords: Pollutants, Phenol, *Burkholderia* sp., *Bacillus pumilus*, Biodegradation, Parameter optimization, Plackett-Burman Design, Central composite design, Haldane model, Immobilized cells.

INTRODUCTION
AND
LITERATURE REVIEW

Chapter-1

Introduction and Literature Review

Water is vital for all forms of life and it covers 71% of earth's surface. Water pollution is a major and common problem faced today. Water pollution is the presence of foreign materials (organic, inorganic and radiological) which are responsible for reduction in the quality of water. Industries use water for variety of purposes like manufacturing goods, heating, cooling, as carrier of raw materials and as a solvent, such industrial activities discharge wastewater into rivers, lakes and oceans and thus continuous industrialization increases the water pollution. Industrial wastewater contains various contaminants and pollutants. These pollutants involved inorganic pollutants, heavy metals, organic pollutants, etc. Inorganic pollutants include alkalis, mineral acids, inorganic salts, free chlorine, ammonia, hydrogen sulphide, salts of chromium, nickel, zinc, cadmium, copper, silver etc. Heavy metals include lead, cadmium, mercury, arsenic etc. Organic pollutants include high molecular weight compounds such as sugars, oils and fats, proteins, hydrocarbons, phenols, detergents, and organic acids. Organic pollutants are potential hazardous chemicals for human health and also toxic to aquatic life in the receiving water. As they persist in environment, they bioaccumulate in human and animals tissue. Phenol is one of the widely occurring organic pollutant and often found in effluent discharged from different industries like coking plants, paper and pulp mills, steel industries, oil refineries, and several chemical industries during the processing of resins, plastics, dyes, varnishes, pharmaceuticals and pesticides, etc. (Carron and Afghan, 1989; Arutchelvan et al., 2006; Agarry and Solomon, 2008). Phenol considered being an extremely hazardous substance as it is toxic even at a low concentration (EPA, 2006).

1.1 Phenol and its Toxicity

Phenol is an aromatic organic compound having molecular formula C_6H_5OH. It consists of benzene ring attached to one hydroxyl group (Fig.1.1). Phenol is also called as Carbolic acid, benzenol, monohydroxybenzene, phenic acid, phenyl alcohol, phenyl hydrate, phenylic acid (NIST, 2011). It is a white crystalline solid at room temperature, which exhibits weak acidic properties. Phenol is soluble in water and is quite flammable. It is also soluble in alcohol and other organic solvents. Phenol is naturally obtained from coal tar or as a degradation product of benzene. Synthetically phenol is made by fusing sodium benzene sulfonate with NaOH, or by heating monochlorobenzene with aqueous NaOH under high pressure (Windholz, 1983). The chemical and physical properties of

phenol have been enlisted in table 1.1.

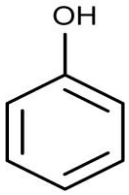


Fig.1.1. Structure of Phenol

Table 1.1: Properties of phenol (IS, 1972; USEPA, 1979)

Physical state	Liquid or solid
Colour	Colourless to light pink
Light sensitivity	Darkens slowly on exposure to light
Odour	Characteristically sweet
Hygroscopicity	Hygroscopic
Solubility in water	6.7 g/100 ml at 16°C
Reactivity	Not dangerously reactive
Flammable limits	Lower limit approximately 1.5 percent
Flash point Open-cup Closed cup	 85°C 79°C
Ignition temperature	715°C
Boiling point (760 mm)	180 to 182°C
Melting point	40 to 41°C
Relative density Solid (25°C/4°C) Liquid (50°C/4°C)	 1.071 1.049
Vapour density (Air = 1)	3.24
Threshold limit value (in air)	5 ppm
Threshold (odour)	0.3 ppm
Vapor pressure	0.3513 mm Hg at 25°C

Phenol is used in the manufacture of many products like insulation materials, adhesives, lacquers, ink, dyes, illuminating gases, perfumes, soaps, toys etc (WHO, 1994). It is mainly used as raw material or intermediate during manufacturing of phenolic resins, bisphenol A, adipic acid, alkylphenols, aniline, chlorinated phenols and caprolactam (Barlow and Johnson, 2007). It is used in some commercial disinfectants, antiseptics, lotions and ointments. Phenol has some medical and pharmaceutical applications including topical anesthetic and ear drops, sclerosing agent. It is also used as a neurolytic agent, applied in order to relieve spasms and chronic pain (Wood, 1978). It is used in dermatology for chemical face peeling. Phenol may be converted into xylenols, alkylphenols, chlorophenols, aniline, and other secondary intermediates in the production

of surfactants, fertilizers, explosives, paints and paint removers, textiles, rubber and plastic plasticizers and antioxidants and curing agents (Busca et al., 2008)

Phenol has been placed in the list of priority pollutants by the U.S. Environmental Protection Agency (USEPA, 1979). Dermal exposure to liquid phenol or to concentrated phenol vapour can result in inflammation and necrosis of the skin (USEPA, 2002). Symptoms of acute toxicity in humans include irregular breathing, muscle weakness and tremors, loss of coordination, convulsions, coma, and respiratory arrest at lethal doses and chronic exposure results in anorexia, progressive weight loss, diarrhea, vertigo, salivation, major damage to the liver, kidneys and eyes, and a dark coloration of the urine (USHHS, 1993; ATSDR, 2008).

1.2 Environmental Pollution due to Phenol Contamination:

Phenol is one of the most common organic water pollutants, because it is toxic even at low concentrations. Due to high water solubility, phenolic compounds lead to widespread contamination of river, lake, estuarine, and other aquatic environments. The effluent from various industries such as pulp and paper, oil refineries, polymeric resins, plastics, steel plants, insecticides, pesticides, textile, dyes, coal processing, pharmaceutical, etc. consist of phenolic compounds as their most important constituents (Pazarlioglu and Telefoncu, 2005).

A large amount of phenol is released in the effluents of the paper mill industry. The primary substrate used in the industry is wood which has three basic components such as cellulose, hemicellulose and lignin. Out of the lignin is a complex phenylpropanoid polymer that provides strength and support to the cellulose and hemi-cellulose structure of the wood. When the left over substrate after processing is released in to effluent, the lignin on degradation produces monomeric phenol which is around 51% of the total composition of the lignin. During bleaching of the pulp, a large amount of the phenolic compounds were also released in to the effluents.

Petroleum hydrocarbon pollution may arise from oil well drilling production operations, transportation and storage in the upstream industry, and refining, transportation, and marketing in the downstream industry. Spilled petroleum hydrocarbons in the environment are usually drawn into the soil. Poor miscibility of crude oil accounts for its accumulation on the surface of ground water and this may migrate laterally over a wide distance to pollute other zones far away from the point of pollution. Toxic components in

oil may also exert their effects on man through inhibition of protein synthesis, nerve synapse function, and damage to plasma membrane (Prescott, et al., 1996).

Environmental Protection Agency set the limits for concentration of phenol in wastewater discharge are 0.5 mg/l for surface waters and 1 mg/l for the sewerage system (Shailubhai, 1986). Table 1.2 enlists various industrial operations and the concentration of the phenol in the effluent generated from them.

Table 1.2 Phenol concentrations in industrial effluents (Busca et al. 2008)

Industry	**Phenol Concentration (mg L^{-1})**
Coking operations	3900
Coal processing	6800
Petrochemicals	1220
Pulp and paper	1600
Gas production	4000
Refineries	500
Pharmaceuticals	1000
Benzene manufacturing	50
Textiles	150
Phenolic Resin Production	1600
Coal Conversion	7000

1.3 Treatment methods for phenolic effluents

Several methods with different removal performance and cost levels are available for phenol removal (Alper and Beste, 2005). These are mainly divided as physico-chemical and biological methods.

1.3.1 Physico-chemical methods:

Several physico-chemical methods such as chemical oxidation, adsorption, extraction, pervaporation etc are used for treatment of wastewater containing phenol.

1.3.1.1 Chemical Oxidation:

Chemical oxidation is the process in which one or more electrons transfer from the oxidant to the targeted pollutant, causing its removal. Various Chemical oxidizing agents are used for removal of phenol. Air, chlorine, ozone, and other chemical oxidizing agent's convert phenol in hydroquinone and then quinone (Yavuz et al., 2007). Sin et al. (2011) used TiO_2 deposited on granular activated carbon (TiO_2/GAC) for photocatalytic degradation of phenol. They investigated effects of photocatalyst loading, initial substrate concentration and addition of an oxidizing agent as H_2O_2 on phenol removal. This process is based on the formation of nonselective and highly reactive radicals such

as hydroxyl radicals (•OH), which can attack a wide range of organic pollutants by converting them into carbon dioxide, water and other associated inorganic salts. Rubalcaba et al. (2007) studied Wet air oxidation and Fenton process in batch mode. They also studied catalytic wet air oxidation and H_2O_2- promoted catalytic wet air oxidation processes in a trickle bed reactor

1.3.1.2 Adsorption:

Adsorption is extensively used in treatment of industrial wastewater. Activated carbons are most commonly used as adsorbents for wastewater treatment (Radovic et al., 2000). The application of activated carbon for phenol adsorption is widely studied treatment method (Dabrowski et al., 2005). The adsorption capacity of activated carbon depends upon physical properties of adsorbent and the solution conditions (Busca et al., 2008). Lin and Juang (2009) studied low cost natural adsorbents like coal fly ash, sludge, biomass, zeolites, and other adsorbents for removal of phenol and their removal capacity compared with synthetic resin. They found that the adsorption capacities of the adsorbents depending on the characteristics of the individual adsorbent, the extent of chemical modifications, and the concentrations of solutes.

1.3.1.3 Solvent Extraction:

Solvent Extraction is also known as Liquid–liquid extraction. It is an effective separation method and it employs partitioning of a solute between two immiscible phases i.e. typically an organic solvent and an aqueous solution (Fan et al., 2008). Several organic solvents such as toluene, *n*-hexane, cyclohexane, benzene, ethylbenzene, cumene, acetate esters (ethyl acetate, isopropyl acetate, *n*-butyl acetate, *n*-pentyl-acetate, *iso*-pentyl-acetate, *n*-hexyl acetate, and *cyclo*-hexyl acetate), di-isopropyl ether, methyl-*iso*-butyl ketone used for extraction of phenol from water (Gonzalez et al., 1986; Pinto et al., 2005). Matjie and Engelbrecht (2007) used “Phenosovan” extraction process for removal of phenol from water in gasification plants. They used diisopropyl ether (DIPE) to recover phenol.

1.3.1.4 Membrane pervaporation:

Pervaporation is an energy saving membrane technique used to separate liquid mixtures (Kujawski, 2000). Pervaporation is a recent technology applied to the removal of organics from water. There are several reports have been cited for this technique. Hoshi et al. (1997) investigated the separation of a phenol–water mixture using a polyurethane

membrane by a pervaporation method. Kujawski et al. (2004) reported application of pervaporation to the removal of phenol using composite membranes.

1.3.2 Limitations of Physico-chemical methods:

Physico-chemical methods mentioned above have major drawbacks such as high cost, energy consumption, production of hazardous by-products, low efficiency and applicability for limited concentration range (Faisal et al., 2003; Beristain-Cardoso et al., 2009). Major drawbacks of solvent extraction are contamination of treated water by the solvent and high cost of solvent. In case of adsorption product recovery is expensive, it require high capital cost and generally spent adsorbent considered as hazardous waste. Chemical oxidation in reactor operates at high temperature and high pressure and ultimately huge energy (Jena et al., 2005). Pervaporation require higher capital cost, purified feed, temperature reduction in process reduces the transmembrane flux (Cavalcante, 2000). Most of the industries still apply various physicochemical methods for the treatment of their effluents. But as discussed above, the physicochemical treatments doesn't fully eradicate the substrates from the effluents and hence they get accumulated in the environment which on due passage of time possess threat to natural flora and fauna (Lacorte et al., 2003).

1.3.3 Biological Treatment Methods:

Development of technology that emphasizes detoxification and degradation of phenol without the above mentioned drawbacks has become the focus of the research. Biological treatment with pure and mixed microbial strains is considered to be an attractive and efficient alternative for the treatment of contaminated wastewaters containing recalcitrant substances such as phenolics since it produces no toxic end products and it is cost effective (Monteiro et al., 2000; Banerjee et al., 2001; Abuhamed et al., 2004; Kumar et al., 2005). Biological process is attractive because microorganisms break down or transform organic pollutant to innocuous substance that leads to complete mineralization of the substrate (Annadurai et al., 2002; Sa and Boaventura, 2001).

1.4 Biodegradation of Phenol

Biodegradation is a process by which microbial organisms transform the structure of chemicals introduced into the environment through metabolic or enzymatic action (USEPA, 2009). A large number of natural and synthetic organic compounds are biodegradable by microorganisms as part of their normal metabolism for energy and

growth. A portion of the organic material, serving as a primary electron and energy source, is converted to oxidized end products through oxidation/reduction reactions. The other portion of the organic carbon is synthesized into cellular material (Basha et al., 2010).

1.4.1 Mechanism of Phenol Biodegradation

Phenol is utilized by aerobic and anaerobic microorganisms. Microorganisms degrade phenol through the action of variety of enzymes. These enzymes may include hydroxylases, oxygenases, peroxidases, tyrosinases, laccase and oxidases (Nair et al., 2008).

1.4.1.1 Aerobic Biodegradation:

In the first step of the aerobic pathway for the biodegradation of phenol (Fig.1.2), phenol hydroxylase uses molecular oxygen to add it to a second hydroxyl group in ortho-position (Basha et al., 2010). The resulting catechol molecule can then be degraded via two alternative pathways (ortho or meta cleavage) depending on the responsible microorganism. The ortho cleavage pathway is also known as β-ketoadipate pathway. The intermediates from both the ortho and meta cleavage pathway are further metabolized to Krebs cycle intermediates. The organisms which utilize phenol by aerobic pathway are *Acinetobacter calcoaceticus, Pseudomonas fluorescens* (Kang and Park, 1997), *Streptococcus* sp. (Mohite and Jalgaonwala, 2011), *Candida tropicalis* (Tuah et al., 2009), *Comamonas testosteroni* (Arai et al., 2000) etc.

1.4.1.2 Anaerobic Biodegradation:

Phenol biodegradation via anaerobic pathway (Fig.1.3) is less advanced than the aerobic process. Some workers reported anaerobic biodegradation of phenol by sludge (Boyd et al., 1983; Battersby and Wilson, 1989). The organisms capable of degrading phenol under anaerobic conditions were *Thauera aromatica* and *Desulphobacterium phenolicum* (Basha et al., 2010). In this pathway the phenol is metabolized to intermediates of Krebs cycle.

Phenol hydroxylase catalyzes the degradation of phenol via two different pathways initiated either by ortho- or meta cleavage pathway. There are many reports on phenol hydroxylase and catechol 2, 3 dioxygenase involved in the biodegradation of phenol (Leonard and Lindley, 1998). Phenol-degrading aerobic bacteria are able to convert phenol into nontoxic intermediates of the tricarboxylic acid cycle via an ortho or meta

pathway (Harwood and Parales, 1996).

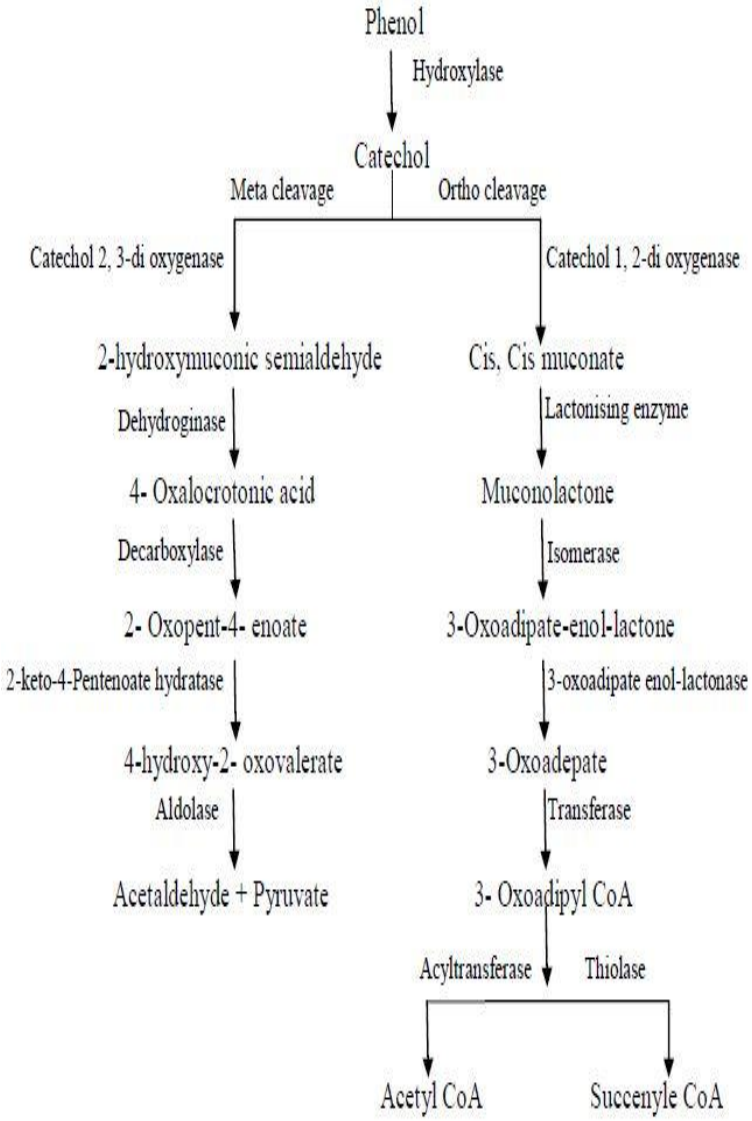


Fig.1.2. Aerobic pathway for phenol degradation (Basha et al., 2010).

The monooxygenation of the aromatic ring constitutes the first step in the biodegradation of many phenolic compounds. This process is carried out by flavoprotein monooxygenases, which use electrons of NADPH to activate and cleave a molecule of oxygen through the formation of an intermediate flavin hydroperoxide and enable the incorporation of an oxygen atom into the substrate (Moonen et al., 2002). These reactions can be catalyzed by a single polypeptide chain or by multicomponent enzymes (van Berkel et al., 2006). It has been reported as a class of monooxygenases, consisting of a small reductase component that uses NADPH to reduce a flavin that diffuses to a large oxygenase component that catalyzes the hydroxylation of aromatic substrate (van Berkel et al., 2006).

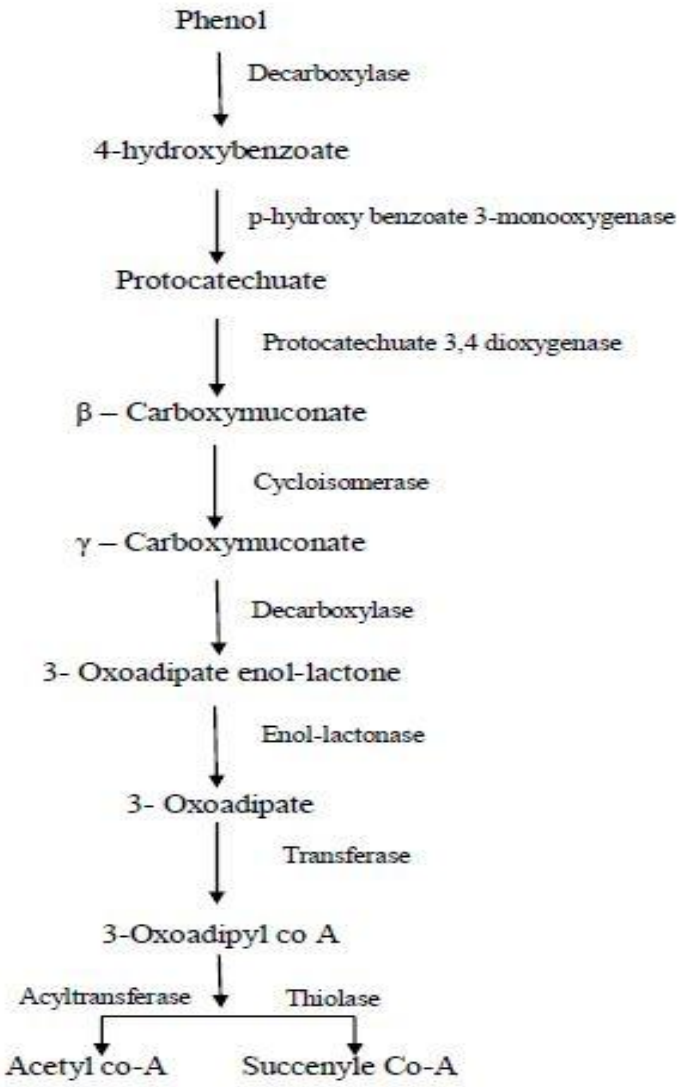


Fig.1.3. Anaerobic pathway for phenol degradation (Basha et al., 2010).

1.5 Biodegradation Studies using Indigenous Microbes

Due to widespread distribution of phenol in the environment, some microorganisms adapted to use the compound both as carbon and energy source. A number of microorganisms have been reported to degrade phenol at various concentrations. Degradation of phenol occurs as a result of the activity of a large number of microorganisms. Bacteria are often the dominant hydrocarbon degraders. These involved the genera Pseudomonas, Achromobacter, Flavobacterium, Halomonas, Bacillus, Nocardia, Arthrobacter, Alcaligenes, Paenibacillus, Azoarcus and Streptococcus etc. The various species of bacteria studied for their phenol degradation ability, such as: *Pseudomonas cepacia* (Folsom et al, 1990), *Acinetobacter calcoaceticus* (Paller et al, 1995), *Bacillus* sp. (Ali et al., 1998), *Pseudomonas putida* (Bandyopadhyay et al, 1998), *Alcaligenes* sp (Baek et al., 2001), *Bacillus pulvifaciens* (Faisal et al., 2003),

Xanthobacter flavus (Nagamani et al., 2009), *Bacillus pumilus* (Gayathri and Vasudevan, 2010), *Pseudomonas aeruginosa* (Silambarasan et al., 2010), *Acinetobacter calcoaceticus* (Yamaga et al., 2010), *Paenibacillus thiaminolyticus* and *Bacillus cereus* (Chandra et al., 2011), *Pseudomonas putida*. (Mahin et al 2011), *Streptococcus* sp. (Mohite and Jalgaonwala, 2011), *Staphylococcus aureus* (Naresh et al., 2012), *Alcaligenes faecalis* (Kumar et al., 2013). The phenol degradation by fungi has been reported by various researchers: *Fusarium* sp. (Santos and Linardi, 2004), *Candida albicans* (San-chin et al., 2005), *Candida tropicalis* (Zhou et al., 2011) etc.

Gurujeyalakshmi and Oriel (1988) have isolated *Bacillus stearothermophilus* BR219 from river sediment and they found that it degrades 15 mM phenol at a rate of 0.85 µmol/h (4 x 10^6 cells). They partially characterized phenol hydroxylase and found that solubilized phenol hydroxylase was NADH dependent, showed a 55°C temperature optimum for activity, and was not inhibited by 0.5 mM phenol.

Kotturi et al. (1991) have studied cell growth and phenol degradation kinetics at 10°C for a *Pseudomonas putida* Q5. They have performed batch mode experiments for initial phenol concentrations, ranging from 14 to 1000 mg/1. They have fitted experimental data by non-linear regression to the integrated Haldane substrate inhibition growth rate model and determined values of the kinetic parameters.

Gunther et al. (1995) have isolated five strains belong to the genus Pseudomonas and two to the genus Bacillus from an aquifier contaminated with phenolic compounds. They have identified most active isolate was *Bacillus pumilus*. They found that the cells of this strain precultured on phenol were able to utilize para-cresol as sole carbon source via the oxidation of the methyl substituent and intradiol ring cleavage of the resulting protocatechuic acid and that led to 4-methylmuconolactone as dead end product and cells precultured on phenol were able to co-oxidize meta- as well as ortho-cresol to 3-methylcatechol, which was cleaved via an intradiol ring fission, finally leading to the dead end-product 2-methylmuconolactone.

Ali et al. (1998) have isolated thermophilic *Bacillus* sp. capable of degrading phenol as the sole carbon from sewage effluent. They found that the *Bacillus* strain Cro3.2, was capable of degrading phenol, o-, m-, and p-cresol via the meta-pathway and tolerated phenol at concentrations up to 0.1% (w/v) without inhibition of growth at optimum temperature of 50–60°C.

Balan et al. (1999) have used *Pseudomonas pictorum* (NICM-2077) an effective strain used in the biodegradation of phenol. They have investigated the effect of glucose, yeast extract, $(NH_4)_2SO_4$ and NaCl on phenol degradation. They have developed Artificial Neural Network (ANN) Model to predict phenol degradation. Then they have compared network model with a Multiple Regression Analysis model (MRA) arrived from the same training data. Further, they have used these two models to predict the percentage degradation of phenol for a blind test data.

Bastos et al. (2000) have isolated *Alcaligenes fecalis* from Amazonian Forest soil. They have performed assays for intracellular and extracellular enzymes and found that this microorganism degrades phenol via Meta cleavage pathway. They found that the isolated strain degrades phenol concentration 700 mg/l within 96 h at pH 7 and 29^{o}C.

Alva and Peyton (2003) have studied the effect of pH and salinity on the biodegradation of phenol by the haloalkaliphilic bacterium *Halomonas campisalis*. They have found that phenol degraded as a source of carbon and energy at pH 8-11 and 0-150 g/l NaCl. They have identified metabolic intermediates catechol, cis,cis-muconate, and (+)-muconolactone and thus they have concluded that phenol was degraded via the β-ketoadipate metabolic pathway.

Yang and Lee (2007) have isolated two phenol-degrading strains from enriched mixed cultures and identified as *Pseudomonas resinovorans* strain P-1 and *Brevibacillus* sp. strain P-6. They have found that optimum growth temperatures for *P. resinovorans* and *Brevibacillus* sp. were 31 and 39^{o}C respectively. They have investigated that when the initial phenol concentration was lower than 600mg/l, *P. resinovorans* could degrade phenol completely within 57.5 h while *Brevibacillus* sp. could remove phenol completely within 93.1 h when the initial phenol concentration was lower than 200 mg/l.

Banerjee and Ghoshal (2010a) have isolated *Bacillus cereus* MTCC9817 strain AKG1 and *B. cereus* MTCC9818 strain AKG2 from petroleum refinery and oil exploration site, respectively. They have found that the bacteria are able to degrade phenol of concentration as high as 2000 mg/L. They have observed that the maximum degradation rate at an initial phenol concentration of about 800 mg/L for the strain AKG1 and about 200 mg/L for the strain AKG2. They also investigated that the strains degrade phenol via meta-cleavage pathway through formation of 2-hydroxymuconic semialdehyde (2-HMSA) as an intermediate product.

Literature study revealed that a considerable amount of work has been done on the biodegradation of phenol and identification of the genes responsible for encoding the enzymes involved in degradation pathways. Microorganisms isolated from similar environmental source responds differently even to the same substrate. This may be due the reason that the microbes isolated from different ecosystem have different growth conditions and follows different metabolic pathway for the degradation of the substrate. Hence these microbes exhibit difference in degradation efficiency and the tolerance potentiality towards the same substrate.

1.6 Optimization of parameters for enhancement of phenol biodegradation

Optimization of microbial growth conditions, particularly physiological and chemical parameters (medium components) are of primary importance in the development of any biodegradation process. The degradation efficiency of the microbes is maximum when the process is carried out under optimum growth conditions. There is broad range of modeling and optimization methodologies, which vary from one factor at a time (OFAT) to complex statistical designs such as Plackett - Burman design, Central composite design (CCD) and Box - Behnken Design (BBD) (Singh and Srivastava, 2013). Single variable optimization methods (One factor at a time) might cause misinterpretation of results as interaction between different factors is overlooked (Abdel-Fattah et al., 2005). On the other hand statistical experimental designs can collectively optimize all the affecting parameters to eliminate the limitations of a single-factor optimization process (Zhou et al., 2011).

In statistical design approach, optimization involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model (Annadurai et al., 2008).

Ryan (2007) gave few desirable criteria for an experimental design as follows:

- The design points should exert equal influence on the determination of the regression coefficients and effect estimates.
- The design should be able to detect the need for nonlinear terms.
- The design should be robust to model misspecification since all models are wrong.

- Designs in the early stage of the use of a sequential set of designs should be constructed with an eye toward providing appropriate information for follow- up experiments.

Coleman and Montgomery (1993) list seven steps that should be made in designing an experiment: (1) recognition and statement of the problem, (2) choice of factors and levels, (3) selection of response variable, (4) choice of experimental design, (5) conduction of experiment, (6) data analysis, and (7) conclusions and recommendations. Statistical experimental designs methods are widely used in industry as an important part of product realization process. Their applications includes the design and development of new products, the improvement of existing product designs, evaluation of material properties, and the design and development of manufacturing process (Montgomery and Jennings, 2006). Industries such as semiconductors and electronics, aerospace, automotive, biotechnology and pharmaceuticals, medical devices, chemical and process industries are all examples where experimental design methodology has resulted in shorter design and development time for new products. Statistical experimental designs also used in research with primary goal are to show the statistical significance of an effect that a particular factor exerts on the dependent variable of interest.

1.6.1 Response surface methodology (RSM):

Response surface methodology was initially developed and described by Box and Wilson (1951). Response surface methodology (RSM) is collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes.

Box and Draper (1987) gave list of desirable properties for response surface designs:

- Satisfactory distribution of information across the experimental region- Rotatability.
- Fitted values are as close as possible to observed values- minimize residuals or error of prediction.
- Good lack of fit detection.
- Internal estimate of error.
- Constant variance check.
- Transformations can be estimated.
- Suitability for blocking.
- Sequential construction of higher order designs from simpler designs.
- Minimum number of treatment combinations.

- Good graphical analysis through simple data patterns.
- Good behaviour when errors in settings of input variables occur.

RSM have extensive application in industries like where several input variables potentially influence some performance measure or quality characteristics of the product or process. This performance measure or quality characteristic is called response (Myers et al., 2009). The input variables are generally called independent variables (Montgomery, 1997). The field of response surface methodology consists of the experimental strategy for exploring the space of process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and process variables. These designs provide information about direct effects, pair wise interaction effects and curvilinear variable effects (Myers and Montgomery, 1995).

Myers et al. (2009) reported that most of the applications of RSM are sequential in nature. At first experiments are designed to investigate the independent variables in order to eliminate the unimportant ones. This type of experiment is generally called a screening experiment. Screening experiments are referred as phase zero. For screening of factors, it is sufficient to identify main effects of significant factors and hence factorial designs are the basis of most of the screening experiments. Plackett-Burman design is generally used for screening phase. After identification of the significant independent variables, phase one of the response surface study begins. In this phase, the main objective is to determine if the current levels of the independent variables result in a value of the response that is near the optimum or if the process is operating in some other region that is might be remote from the optimum. Various response surface designs like central composite design and Box-Behnken design are preferred for phase one.

1.6.1.1 Plackett-Burman Design:

Plackett-Burman design was developed by Plackett and Burman in 1946. It is two level fractional design for studying up to k= N-1, where k are variables and N is the number of runs. Plackett-Burman designs with N= 8, 16, 32 etc. i.e. power of two are called as geometric designs. The perfect confounding is the advantage of this design. Plackett-Burman designs with N = 12, 20, 24, 28 and 36 where N is multiple of 4, are often called nongeometric Plackett-Burman designs These designs have complex alias structures and hence this design generally preferred for screening of significant factors (Myers et al.,

2009). This design is resolution III design and hence might include only main effects (Mathews, 2010).

1.6.1.2 Central composite design (CCD):

Central composite design (CCD) is the most popular types of second order response surface designs. It is designed to estimate the coefficients of a quadratic model. All point descriptions will be in terms of coded values of the factors. It can be run sequentially and it involves the use of a two-level factorial or fraction (resolution V) combined with the axial or star points. It gives reasonable information for testing lack of fit and not involving large number of design points (Myers et al., 2009).

A CCD has three groups of design points:

- Two-level factorial or fractional factorial design points: The two-level factorial part of the design consists of all possible combinations of the +1 and -1 levels of the factors. For the two factor case there are four design points: (-1, -1) (+1, -1) (-1, +1) (+1, +1).
- Axial points (sometimes called "star" points): The star points have all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha. For a two factor problem, the star points are: (-Alpha, 0) (+Alpha, 0) (0, -Alpha) (0, +Alpha)
- Center points: Center points are points with all levels set to coded level 0 i.e. the midpoint of each factor range: (0, 0) Center points are usually repeated 4-6 times to get a good estimate of experimental error (pure error). Replicated center point provides excellent prediction capability near the center of the design space and provides information about the existence of curvature in the system.

The flexibility in the use of central composite design mainly depends upon the selection of value of alpha and number of center runs. The choice of alpha mainly depends upon the region of operability and region of interest (Myers et al., 2009). There are mainly five types of alpha values can be entered: a) Rotatable (k<6): It is the default setting for up to 5 factors and this creates a design that has the standard error of predictions equal at points equidistant from the center of the design; b) Spherical: This puts all factorial and axial points on the surface of a sphere of radius = $\sqrt{k}$; c) Orthogonal Quadratic: This provides alpha values that allow the quadratic terms to be independently estimated from the other terms; d) Practical (k>5): This is the default for designs that have 6 or more

factors and it is the 4th root of the number of factors; e) Face Centered: The axial points are pulled into the faces of the cube at +/- 1 levels. This produces a design where each factor only has 3 levels. It is also possible to give any desired value of alpha and thus it is not confined to above mentioned types of alpha values.

Sheeja and Murugesan (2002) have developed model by using RSM that indicates maximum phenol degradation is a function of independent variables: pH, temperature, initial phenol concentration and diameter of immobilized beads. They have reported that the free cells completely degrade phenol concentration of $\leq$1g dm^{-3} while cells immobilized in alginate beads degrade phenol concentration $\leq$ 2g dm^3. They have observed maximum phenol degradation at pH 7 ± 1 and temperature 33^oC. They have found that the model fits the second order equation well with correlation coefficients of 0.9999 and 0.9993 for *Pseudomonas pictorum*-alginate beads and activated carbon-*Pseudomonas pictorum* – alginate beads respectively.

Annadurai et al. (2008) have used RSM to optimize medium composition for degradation of phenol by *Pseudomonas putida* (ATCC 31800). They have developed a mathematical model to show effect of each medium composition and their interactions on the biodegradation of phenol. They have found that biodegradation of phenol is pH dependent and maximum phenol degradation achieved at pH 7 and temperature 30^oC and phenol concentration 0.2 g/l. They carried out the design of experiments for analysis using the Design Expert by Stat Ease Inc (version 7).

Agarry et al. (2008) have studied phenol degradation by using *Pseudomonas aeruginosa*. They have studied three process parameters i.e. temperature (25– 45^oC), aeration (1.0 – 3.5 vvm) and agitation (200 – 600 rpm) for optimization of phenol biodegradation. They have used response surface methodology to get significant effects and the interactions between the three parameters. They have employed 2^3 full-factorial central composite designed followed by multistage Monte-Carlo optimization technique for experimental design and analysis of result. They have obtained optimum process conditions for maximizing phenol degradation (removal) as follows: temperature 30.1^oC, aeration 3.0 vvm, and agitation 301 rpm. They have found the maximum removal efficiency of phenol (94.5%) at the optimized process conditions.

Agarry et al. (2010) have used one variable at a time bioprocess design and RSM to evaluate effects of aeration, agitation and temperature on phenol degradation by *Pseudomonas fluorescence*. They have selected factors for optimization as: (25-45^oC),

aeration (1.0-3.5 vvm), and agitation (200-600 rpm). They have used 2^3 full factorial central composite design for optimization of phenol degradation. They have found the second order polynomial regression model with R^2= 0.9647 and the optimum conditions for maximum phenol degradation as: temperature 30^oC, aeration 3 vvm and agitation 300 rpm with maximum phenol degradation rate as 60.7%.

Lakshmi et al. (2011) have studied phenol degradation by *Pseudomonas aeruginosa* (NCIM 2074). They have performed experiments with variables as carbon source (glucose), inorganic nitrogen (ammonium chloride) and metal ion concentration (zinc ion). They have used a 2^3 full factorial central composite design combining with Response Surface Methodology (RSM) to optimize the process parameters for the degradation of phenol. They have found a second order polynomial regression model with an R^2 value of 0.9669 and an F-value of 32.52295. They have observed that the maximum degradation of phenol was estimated up to 80.45% at optimized conditions.

Sridevi et al. (2011) have investigated phenol biodegradation in a batch reactor using *Pseudomonas putida* (NCIM 2102). They have studied chemical parameters like carbon source (glucose, galactose, D-xylose, fructose and sucrose), inorganic nitrogen source (ammonium sulfate, sodium nitrate, disodium phosphate and sodium phosphate) and metal ions (Manganese, lead, cobalt and Cu (II)) at various concentrations for optimization of phenol degradation. They have used Statistica (version 6.0) for development of quadratic model. They have found the optimum conditions for maximum phenol degradation at a glucose concentration of 0.8229 g/l, $(NH_4)_2SO_4$ (Ammonium sulfate) concentration of 1.5183 g/l and metal ion concentration (Mn^{2+}) of 0.0195 g/l. They have obtained maximum 98.24 % phenol degradation at these optimized parameters.

Zhou et al. (2011) have used statistical experimental designs to optimize the process of phenol degradation by *Candida tropicalis* Z-04, isolated from phenol-degrading aerobic granules. They have used Design-Expert Version 7.0.1 (Stat-Ease Inc., Minneapolis, USA) for designing of experiments. They have identified most important factors influencing phenol degradation ($p < 0.05$) by a two-level Plackett-Burman design with 11 variables and those were yeast extract, phenol, inoculum size, and temperature. They have further used steepest ascent method to determine the optimal regions of these four significant factors. Then they performed central composite design (CCD) experiments and response surface analysis for these significant variables. They have observed the

maximum phenol degradation (99.10%) under the optimum conditions of yeast extract 0.41 g/l, phenol 1.03 g/l, inoculum size 1.43% (v/v) and temperature 30.04°C.

Balamurugan et al. (2012) have applied statistical design for optimization of phenol degradation by *Aspergillus fumigates* (MTCC No.343) in batch reactor. They have used Design Expert software for designing of experiments. They have studied effect of initial phenol concentration, pH, temperature and inoculum size for the on Removal Efficiency (RE) of phenol and optimized these factors using Response Surface Methodology (RSM). They have used Central Composite Design (CCD) and performed 31 experiments for the four test variables. They have found 95% phenol RE at the optimized conditions as follows: initial phenol concentration 300 mg/l, pH - 7, temperature 28°C and inoculum size 5%.

Suhaila et al. (2013) have used Response surface methodology (RSM) to optimize medium composition and culture condition for enhancement of growth of *Rhodococcus* UKMP-5M and phenol degradation rate in shake flask cultures. They have used Design-Expert Version 6.0.6 (Stat-Ease Inc., Minneapolis, USA) for generating experiments and analyzing data. They have found the temperature, phenol concentration and $(NH_4)_2SO_4$ concentration were the most significant factors for growth and phenol degradation. They have used Central composite design (CCD) for optimization of these parameters with growth, and degradation rates used as the responses. They have found that 0.5 g/L phenol, 0.3 g/l $(NH_4)_2SO_4$ and incubation at 36°C greatly enhances growth of *Rhodococcus* UKMP-5M. They also observed that the degradation rate increases at 0.7 g/l phenol, 0.4 g/l $(NH_4)_2SO_4$ and incubation at 37°C and at these conditions the time for degradation of 1 g/l phenol in the culture reduces from 48 h to 27 h.

Parameters for phenol degradation must be optimized in order to subjugate phenol concentration within acceptable level. The above literature study revealed that parameters like initial phenol concentration, pH, temperature, inoculum size and concentration of various medium components induce important effect on phenol degradation ability of the microbe. Hence it is necessary to optimize these parameters for enhancement of phenol degradation.

1.7 Kinetics of Phenol degradation

Evaluation of the biokinetic constants is significant for understanding the capacities of the microorganisms for the degradation and for the operation of biological reactors. The various kinetic substrate utilization and inhibition models have been studied for

microbial growth on phenol (Colvin and Rozich, 1986; Bandyopadhyay et al., 1998; Marrot et al., 2006). Kinetic study of phenol degradation is extensively studied by various researchers.

Goudar et al. (2000) have studied phenol degradation in batch experiments using an acclimated inoculum of mixed culture and initial phenol concentrations ranging from 0.1 to 1.3 g/l. They have used a generalized substrate inhibition model based on statistical thermodynamics to describe the dynamics of microbial growth in phenol. Further they have reduced the generalized substrate inhibition model to a form that is analogous to the Andrews equation and they found the biokinetic parameters: maximum specific growth (μ_{max}) 0.251h^{-1}; saturation constant (K_s) 0.011 g/l and inhibition constant (K_i) 0.348 g/l.

Peyton et al. (2002) have isolated Halophilic bacterial cultures from Salt lake basin. They have used 10% NaCl for enrichment of culture. They have modeled phenol degradation and corresponding cell growth by zero order kinetics with respect to phenol concentrations and first order kinetics with respect to cell concentration. They found that, at an initial phenol concentration 50 mg/l specific growth rates were ranges between 0.22 to 0.32 h^{-1} for mixed culture. They have observed that one of the cultures can degrade phenol concentration 320 mg/l within 68 h at 30^{o}C and pH 7. For this culture, they have found the specific growth rate as 0.09 to 0.22 h^{-1}. They have observed that specific growth rates decreases as initial phenol concentration increases

Faisal et al. (2003) have isolated *Bacillus pulvifaciens* from various contaminated soil. They have immobilized cells on loofa sponge and performed phenol removal study in airlift Bioreactor A and Bioreactor B containing pure culture and mixed culture respectively with initial phenol concentration 30 mg/l. For 27 hrs incubation in airlift bioreactor, they found that 99.8% and 84.2% phenol removed by isolated bacteria and activated sludge culture respectively. They investigated kinetic parameters on the basis of Monod growth model and found these parameters as: μ_{max} (1/h) = 0.21 and K_s (mg/l) = 1.92 for pure culture and for mixed culture they found kinetic parameters as: μ_{max} (1/h) = 0.28 and K_s (mg/l) = 5.68.

Rigo and Alegre (2004) have isolated *Candida parapsilopsis* from phenol containing industrial wastewater. They have found that Haldane equation to be fit for each batch culture with different initial phenol concentration. They have used non linear least squares regression analysis and found the X^2 coefficient as 0.00003. They have obtained

the Haldane parameters as: μ_{max}=0.174 ± 0.014 h^{-1}, K_s = 11.2 ± 3.48 mg/l and K_i=298 ± 48.4 mg/l.

Kumar et al. (2005) have investigated biodegradation of phenol and catechol by *Pseudomonas putida* (MTCC 1194) in shake-flask experiments at 29.9±0.3^oC and pH 7.1. They have acclimatized this bacterial strain to the concentrations of 1000 and 500 mg/l for phenol and catechol, respectively for a period of three months. They have found the longer lag period for higher concentration of phenol and catechol. They have observed that the initial phenol concentration of 1000 mg/l and initial catechol concentration of 500 mg/l completely degrades within 162 and 94 h, respectively. They have used Haldane's growth kinetics model to fit the growth kinetics data and further, for phenol biodegradation they found the μ_{max} = 0.305 h^{-1}, K_s = 129.79 mg/l, and K_i = 36.33 mg/l.

Arutchelvan et al. (2006) have performed kinetic study of phenol degradation by *Bacillus brevis* isolated from phenol–formaldehyde resin manufacturing industrial wastewater. They have used Haldane kinetic model to describe cell growth with kinetic constants and these were obtained as: μ_{max} = 0.026–0.078 h^{-1}, K_s = 2.2–29.31 mg/l and K_i = 868.0–2434.7 mg/l. Their result indicates the inhibition effect of phenol increases as its concentration increases.

Juang and Tsai (2006) have studied kinetics of biodegradation of single phenol and sodium salicylate (SA) and their binary mixtures in water by *Pseudomonas putida* CCRC 14365 at 30$^\circ C$ and pH 7.0. By applying Haldane model, they have found the kinetic parameters for phenol degradation as: μ_{max} = 0.245h^{-1}, K_s = 0.129 mM and K_i = 12.6 mM and for SA as: = 0.137h^{-1}, K_s = 0.111 mM and K_i = 5.21 mM. They have observed phenol degrades rapidly than the SA, since the maximum cell growth rate (μ_{max}) on phenol is larger.

Saravanan et al. (2008) have performed biodegradation study of phenol by mixed microbial culture, isolated from sewage treatment plant, in batch mode shake flasks. They have used Monod, Haldane and Han–Levenspiel models for growth kinetics study of culture. They found that substrate inhibition kinetics and the specific growth rate were fitted to Haldane and Han–Levenspiel models. They have found that the growth kinetics parameter values for Monod model: μ_{max} = 0.37h^{-1} and Ks = 144.68 mg/l; for Haldane model: μ_{max} = 0.3085h^{-1}, K_s = 44.92 mg/l and K_i = 525 mg/l; for Han–Levenspiel model: μ_{max} = 0.4029 h^{-1} and K_s = 110.93 mg/l. They have observed that biokinetic constants

estimated using these models showed good potential of the mixed microbial culture in phenol degradation

Banerjee and Ghoshal (2010b) have studied degradation of phenol by pure cultures *Bacillus cereus* MTCC 9817 strain AKG1 and *B. cereus* MTCC 9818 strain AKG2 is in batch mode for phenol concentrations in the range of 100–2000 mg/L with an interval of 100 mg/L. They have studied growth kinetic analysis of strains by applying kinetic models like Yano model, Haldane model, Aiba model, Webb model, Edward model. They have found that modeling of the Haldane inhibitory model fits the experimental data fairly well for both the strains and they have estimated kinetic parameters: q_{max} = 27.85h^{-1}, K_s = 59,150 mg/l and K_i = 2.411 mg/l.

Dey and Mukherjee (2010) have collected phenol degrading mixed culture from coke oven industry. They have found that the culture was able to degrade phenol upto 700 mg/l. They have observed that specific growth rate of microorganisms and specific substrate degradation rate increased up to 300 mg/l of initial phenol concentration and then decreases. They have fitted experimental growth kinetic data to various kinetic models by MATLAB 7.1©.They have found that the Haldane model fitted well for phenol degradation (0 -700 mg/l) and the kinetic parameters found as: μ_{max} = 0.3057h^{-1}, K_s = 257.5 mg/l, and K_i = 162.6 mg/l. They have observed that the reasonable kinetic parameters values' indicating the mixed culture is potential culture for phenol degradation.

Essam et al. (2010) have isolated high phenol tolerance *Alcaligenes* sp. TW1 from the activated sludge of the industrial wastewater treatment plant of a Coke company. They have used Haldane kinetics model to growth on phenol as sole carbon and energy source at 25^{o}C and they have obtained kinetic parameters as: μ_{max} = 0.58 h^{-1}, K_s = 10 mg/l, and K_i = 152–550 mg/l and they have found biomass yield coefficient ranged from 0.55 to 0.64 mg dry cell mass/mg phenol for maximum 1200 mg/l phenol concentration.

Among kinetic models reported in literature (Table 1.3), Haldane model best fits to growth data of microbes for phenol biodegradation since phenol is a growth limiting substrate. It is widely accepted for representing the growth kinetics of inhibitory compounds due to its mathematical simplicity and accuracy. Thus, there is scope to evaluate the growth kinetic parameters for isolated strains using Haldane model.

Table 1.3: Kinetic models and their model equations reported for phenol biodegradation

Model	Model equation	Reference
Haldane model	$\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_i}}$	Dey and Mukherjee (2010)
Monod model	$\mu = \frac{\mu_{max} S}{K_s + S}$	Faisal et al. (2003)
Han–Levenspiel model	$\mu = \frac{\mu_{max}[1 - \frac{S}{S_m}]^n}{K_s + S - [1 - \frac{S}{S_m}]^m}$	Saravanan et al. (2008)
Yano model	$q = \frac{q_{max} S_0}{K_s + S_0 + \left(\frac{S_0^2}{K_I}\right)[1 + (\frac{S_0}{K})]}$	Banerjee and Ghoshal (2010b)
Aiba model	$q = \frac{q_{max} S_0}{K_s + S_0} \exp(\frac{-S_0}{K_I})$	
Webb model	$q = \frac{q_{max} S_0 [1 + (S_0/K)]}{S_0 + K_s + (S_0^2/K_I)}$	
Edward model	$q = q_{max} S_0 [\exp\left(\frac{S_0}{K_I}\right) - \exp(\frac{S_0}{K_s})]$	

1.8 Biodegradation studies using immobilized cell

Biodegradation of phenol using pure and mixed cultures of suspended bacteria has been widely investigated. However at higher initial concentration of phenol the growth as well as the degradation activity of the free cells (suspended cells) gets inhibited due to toxicity. Hence a number of strategies have been developed to overcome the same. Among them, immobilization of cells is effective technique for protecting the microbial cells against inhibition of phenol. The immobilized cells have advantages in comparison with suspended ones and these include the retention in the reactor of higher concentrations of microorganisms, protection of cells against toxic substances and eliminate the costly processes of cell recovery and cell recycle (Dursun and Tepe, 2005). Phenol biodegradation by immobilized cells has been reported by several workers.

Mordocco et al. (1999) have studied continuous degradation of phenol by *Pseudomonas putida* ATCC 11172. They have performed continuous degradation of phenol concentrations as low as 2.5 mg/l to 100 mg/l with immobilized cells. They have observed that the increase in dilution rate increases degradation rate but only to 0.6 h^{-1} beyond which effluent phenol concentration begin to rise. They have achieved the highest degradation rate as 108 mg/l/h. They have found the dilution rates above 0.3 h^{-1}

was better for phenol degradation by the immobilized cell than a free cell. They have also found that the pH 5.5-6.0, temperatures 25°C-30°C and a bead diameter 1-2 mm to be optimum for phenol degradation at low levels.

Gonzalez et al. (2001) have studied biodegradation of phenolic industrial wastewater by immobilized cells of *Pseudomonas putida* ATCC 17484 in fluidized bed bioreactor in batch and continous mode. In batch mode, they have found that phenol concentrations upto 1000 mg/l were degraded with 90% removal efficiency. They have observed that the strain is efficient for phenol degradation in FBB with 500 mg/l d phenol loading rate.

Pazarlioglu and Telefoncu (2005) have studied phenol biodegradation by *Pseudomonas putida* immobilized on activated pumice particles. They have found cell adsorption ratio 91% with Zr-activated pumice. They have observed the biocatalyst completely degrades 1.0 g/l phenol in the batch shaking flasks in 22 h. They have also used these immobilized cells in recycled and continuous mode packed bed bioreactors for phenol degradation. They have found that the biocatalyst can be stored at $4^{\circ}C$ for 6 months without significant decrease in activity.

Karigar et al. (2006) have isolated *Arthrobacter citreus* from Hydrocarbon contaminated site. They have immobilized strain by alginate and agar matrices. On immobilization of this strain, they found that it degrades phenol concentration 500 mg/l within 24 h at $30^{o}C$ and pH 7. Their study shows that the immobilized *Arthrobacter citreus* can efficiently degrade phenol even at higher concentrations.

Massalha et al. (2007) have isolated phenol degrading bacteria cells from compost, which included a mixture of olive mill and piggery solid waste. They have used different low-cost mineral additives (clay and activated carbon) at the immobilization matrix and performed aerobic biodegradation of phenol using isolated microorganism. They have studied influence of a different initial concentration of phenol (400-2000 mg/l) on the rate of biodegradation by free and immobilized cells. Their results shows that immobilized cells tolerates and completely degrades phenol at initial concentrations of 2000 mg/l and higher. They have found that the bead size of 4 mm (diameter) to be optimum to degrade phenol at an initial concentration of ~2000 mg/l. Results obtained by them shows that the bead size significantly affected the biodegradation rate of phenol.

Ying et al. (2007) have isolated *Acinetobacter* sp. strain PD12 from activated sludge of wastewater treatment plant. They have observed that it was capable of degrade phenol concentration 500 mg/l in 9 h as free cells. They immobilized these cells by using polyvinyl alcohol gel and found that these cells degrade 1100 mg/l in 120 h at pH 7.2

and 32^{o}C. They have found that these immobilized cells possess greater storage stability and it can be used for at least 50 cycles. Their results indicates that immobilized *Acinetobacter* sp. strain PD12 have potential application in the treatment of phenol-containing wastewater.

Santos et al. (2009) have performed batch study of phenol (2–30mM) degradation by free cells and by alginate-immobilized cells of *Aureobasidium pullulans* FE13 isolated from stainless steel effluents. They have found that the up to 16mM of phenol concentration, degradation rate by immobilized cells was similar to the degradation rate by the suspended cells. They have observed inhibitory effect at concentrations higher than 16mM of phenol and which was resulting in the decreasing of the phenol degradation rates. They have observed that the immobilized cells remained viable for a longer period and thus increasing the efficiency of phenol degradation. Their results suggest that immobilized cells of *Aureobasidium pullulans* FE13 have potential application in the biodegradation of phenol.

Passos et al. (2010) have performed comparative study of free and encapsulated cells of *Aspergillus* sp. LEBM2.in batch cultures. They have observed that the maximum phenol degradation rates were not significantly different between free and encapsulated cells. But, they found a decrease in adaptation time for encapsulated cells. Encapsulated cells showed maximum phenol degradation rate of 7.71 ± 0.21 mg/l/h for an initial concentration of 500 mg/l. They have found that the presence of a microenvironment is more effective for biodegradation inside encapsulated cells as it is protective and thus reduce abiotic stress.

Ahamad and Kunhi (2011) have studied phenol degradation by *Pseudomonas* sp. CP4 entrapped in agar and calcium alginate beads in batch and continuous mode. In batch mode, they have observed agar-encapsulated cells degrades phenol up to 3000 mg/l as compared to 1500 mg/l by Ca alginate entrapped cells whereas free cells could tolerate only 1000 mg/l at an aeration rate of 1 vol^{-1} vol^{-1} min^{-1}, 30°C and pH 7.0 ± 0.2. In continous process, they have obtained a degradation rate of 200 mg phenol l^{-1} h^{-1} with Ca-alginate entrapped cells while agar-entrapped cells degrades up to 4000 mg phenol l^{-1} in the feed with a maximum degradation rate of 400 mg phenol l^{-1} h^{-1}. They have observed that the *Pseudomonas* sp. CP4 is capable for phenol degradation at high concentrations.

Pishgar et al. (2011) have isolated mixed culture from the effluent of two industries coke oven industry and pulp and paper industry. They entrapped and immobilized

microorganisms in calcium-alginate gel beads. They used inocula concentration 10% (v/v) and performed degradation experiments in batch mode under anaerobic condition at room temperature of about 25°C and initial pH of 7.0 with phenol concentration varied in the range of 70 to 1000 mg/l. They found at phenol concentration of 1000 mg/l, the removal efficiency enhances from 10 to about 40% in the presence of immobilized cells and maximum biodegradation rate happened at phenol concentration of 700 mg/l which was 2.13 and 2.65 mg/l/h for free and immobilized cells, respectively. Results obtained by them shows that the adaptation of immobilized cells leads to slightly shorter time for complete phenol removal in the range of 100-700 mg/l.

Immobilization of microbes enhances degradation efficiency and tolerance. According to the previously available research data, microorganisms immobilized on different support materials such as calcium alginate beads, agar matrices, polyvinyl alcohol gel etc. are showing different degradation efficiency and tolerance level. Among them, calcium alginate is showing advantages as a support, such as good biocompatibility, low cost, easy availability and ease of preparation. Hence there is scope to contemplate the behavior of immobilized isolated microbes on calcium alginate beads for phenol degradation in the present study.

1.9 Scope of the present work

As reported in literature, large number of bacterial strains has been isolated from various contaminated sites. Microbes isolated from diverse ecological sources behave otherwise to the same substrate due to difference in their growth conditions. Bacteria can quickly adapt to the fluctuations in environmental conditions, both physiologically and genetically. Selective enrichment enables specific bacteria to be dominant to grow under desired conditions (Elsas et al., 2006). Paper mills utilizes huge amount of phenolic compounds during the manufacturing processes leading to discharge of vast amount of phenol contaminated effluent in to the environment. Similarly, phenolic compounds are also a major component of crude oil (Field et al., 1940; Onwurah et al., 2007). Oil spillage sites are also a source of phenol contamination of the nearby soil and ground water and hence become a major environmental problem. The microbial communities existing in such contaminated sites are able to tolerate high concentration of phenol. Thus soil contaminated with paper mill effluent and crude oil is a possible source of isolating highly efficient phenol degrading bacteria.

New or even same microorganisms isolated from different ecosystem exhibits different degradation potential. Hence to achieve maximal phenol degradation, the growth parameters are needed to be optimized. With difference in the tolerance and degradation potential, bio-kinetic parameters for the scale up study changes for the microorganism. Thus the bio-kinetic parameters are to be determined for usage of the microbe for large scale treatment of phenol. Each microorganism exhibits a difference in enhanced degradation efficiency when immobilized on a support matrix. Same microbe results in different degradation potential on being immobilized on different support matrix. Thus characterization of the enhancement of tolerance and degradation efficiency of the isolated microbes is necessary for the use of microbes in immobilized cell bioreactors. In view of the above scope, the objective of the current work has been presented in the following section.

1.10 Objectives of the Present Work

The specific objectives of the present research work are as follows:

- Isolation and characterization of phenol degrading bacterial strains from soil contaminated with paper mill effluent and crude oil.
- Parameter optimization for phenol biodegradation by the isolated strains.
- Evaluation of Growth kinetics parameters of isolated strains for phenol biodegradation by using Haldane model.
- Study the degradation behaviour of free and immobilized cells of isolated species at various initial phenol concentrations.

1.11 Layout of the thesis

This thesis contains four chapters: Introduction and literature review, Materials and methods, Results and discussion and Conclusion and future work.

Chapter 1, deals with the introduction, literature review and objectives of present work.

Chapter 2, deals with materials and methodology of independent experimental work performed for the isolated strains.

Chapter 3, discuss about results obtained from identification of isolated strains, optimization study of phenol biodegradation, phenol degradation characteristics of isolated strains at various concentrations, growth kinetic study isolated strains for phenol degradation and phenol degradation study by immobilized cells.

Chapter 4, deals with overall conclusion and future work based on outcomes of present work.

MATERIALS AND METHODS

Chapter-2

Materials and Methods

2.1 Chemicals and Reagents

Pure and analytical grade chemicals have been used in present study. Phenol, Hydrochloric acid and other chemicals have been procured from Merck®, India. Nutrient agar, Nutrient broth, Agar powder (Bacteriological grade) and other media components have been procured from HIMEDIA®, India. Chemicals and kits used for the biochemical characterization of the isolate have been obtained from HIMEDIA®, India.

2.2 Glasswares and Instruments

All glasswares (Test tubes, Conical flasks, Petri dishes, Beakers etc.) used in study have been purchased from Borosil. The instruments and apparatus used throughout study are enlisted in *Appendix (D)*.

2.3 Isolation of phenol degrading bacterial strains

2.3.1 Sample collection:

Soil samples have been collected from treated effluent discharge site of J.K. Paper mill, Rayagada (Odisha) and crude oil spillage site at Haldia Oil refinery, Haldia (West Bengal). Top layer of soil has been removed up to 1-2 cm and the sterile scoops have been used for collection of soil samples. Four subsamples have been taken from each point and mixed in sterile plastic bags. Soil samples have been stored at ambient temperature during travelling.

2.3.2 Growth Medium:

Mineral salt media used for enrichment and screening of phenol degrading strains in the present study is as per Bai et al. (2007) has a composition as follows (mg/l): 400 K_2HPO_4, 200 KH_2PO_4, 400 $(NH_4)_2SO_4$, 100 NaCl, 100 $MgSO_4$, 10 $MnSO_4.H_2O$, 10 $Fe_2(SO_4)_3.H_2O$, 10 $Na_2MoO_4.2H_2O$. Phenol has been used as sole source of carbon and pH maintained at 7. The media sterilization has been by autoclaving while phenol has been sterilized by 0.25 µm syringe filters and added to the medium before inoculation.

2.3.3 Enrichment of phenol degrading strains:

Five gram of each soil sample has been added into the 100 ml mineral salt media containing 100 mg/l phenol as a carbon source and it has been incubated in 250 ml flasks at 30°C and 150 rpm for 48 h. After incubation, soil particles have been allowed to settle

and 5 ml of particle free sample has been inoculated in 100 ml mineral salt media containing 100 mg/l phenol and incubated at 30°C for 48 h at 150 rpm. Same procedure has been repeated for thrice. The enriched samples have been diluted and transferred on sterile petri plates containing mineral salt media having 100 mg/l phenol concentration and after incubation, morphologically distinct colonies have been selected for further screening study. The selected isolates have been purified by repeated streaking on mineral salt media.

2.3.4 Screening of phenol degrading strains:

The method developed by Rigo and Alegre (2004) has been used for screening of phenol degrading strains. All isolates obtained after enrichment have been individually inoculated in mineral salt media containing phenol concentration 200 mg/l and incubated at 30°C and 150 rpm for 48 h. The isolates which showed growth in broth have been transferred on petri plate with 200 mg/l phenol containing minimal salt medium and incubated at 30°C for 48 h. subjected to subsequent increase in the concentration of phenol with an increment of 50 mg/l each time till the complete inhibition of their growth. The strain designated as PS3 isolated from the paper mill site has been found to tolerate up to 1500 mg/l of initial concentration of phenol while the strain designated as OS1 isolated from oil refinery site has been found to tolerate up to 1250 mg/l of phenol. These strains have been selected for further study. Isolated pure cultures have been sub-cultured at an interval of every 15 days and stored at 4°C.

2.4 Identification of isolated phenol degrading strains

The isolated strains PS3 and OS1 have been indentified on the basis of their morphological, biochemical and molecular characteristics. Bergey's manual of determinative of bacteriology was used as a reference to identify the isolates.

2.4.1 Morphological characteristics:

2.4.1.1 Colony morphology:

The isolated strains have been examined for colony morphology: size, shape, colour, margin, opacity, elevation, and textures.

2.4.1.2 Motility:

The drop of overnight incubated bacterial suspension used for hanging drop slide method to observe motility.

2.4.1.3 Gram Staining:

The smear has been prepared from overnight old cultures on glass slide. The smear has been heat fixed and flooded with crystal violet staining reagent for 30 seconds. The slide has been washed gently in stream of tap water for 2 seconds. The slide has been dried and then flooded with Gram's iodine and kept for 1 minute. The slide has been washed with 95% alcohol for 15 seconds and subsequently gently washed with water. After drying the slide has been flooded with safranin for 30 seconds and then slide has been washed gently with water. The dried slide has been observed under oil immersion lens using light microscope.

2.4.2 Biochemical characteristics:

Biochemical characteristics of both the strains independently have been studied by following tests:

2.4.2.1 Catalase test:

Nutrient agar (*Appendix B*) plates have been inoculated with isolated strains and have been incubated at 30°C for 24 hours. After incubation, a loop full of overnight culture were collected and placed on a microscopic slide within a blank petri dish. One drop of 3% H_2O_2 placed onto the organism on the microscope slide. The production of gas bubbles indicates presence of catalase.

2.4.2.2 Oxidase test:

Nutrient agar (*Appendix B*) plates streaked with isolated strains and have been incubated at 30°C for 24 h. After incubation, one to two drops of oxidase reagent has been added to the plates. The reactions have been observed. If the color changes to dark purple within 5 to 10 seconds indicates microorganisms are oxidase positive. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

2.4.2.3 Nitrate reduction test:

Isolated strains have been inoculated individually in nitrate reduction media (*Appendix B*) and inverted Durham tubes added into it and incubated at 30°C for 24 h. The Durham tubes have been observed for gas production. Two drops each of reagents A and B (*Appendix B*) mixed in a small test tube. One ml of the broth culture added to the test tube and mixed well. If the test organism has reduced the NO_3^- to NO_2^-, a red color will

usually appear within 2 minutes, indicating the presence of NO_2^- in the tube. If no color change is seen within 2 minutes, a small amount of powdered zinc need to be added. If the tube turns red after the addition of the zinc, it indicates that unreduced nitrate has been present. Hence it is a negative result but if the medium does not turn red it is positive result.

2.4.2.4 Gelatin liquefaction test:

Nutrient gelatin agar (*Appendix B*) tubes have been inoculated with isolated strains. Tubes have been incubated for 24 h at 30°C and observed for liquefaction of gelatin.

2.4.2.5 Starch hydrolysis test:

Starch agar plate (*Appendix B*) has been inoculated with the isolated strains. Plates have been incubated at 30°C for 48 h. To test the hydrolysis of starch, each plate has been flooded with iodine. The clear zone around colony indicates positive result.

2.4.2.6 Indole test:

The tryptone broth (*Appendix B*) tubes have been inoculated with the isolated strains. The tubes have been incubated for 48 h at 30°C. The 0.3 ml of Kovac's reagent added to each test tube. The formation of a red layer at the top of the culture indicates a positive test. A negative result has a yellow or brown layer at the top of culture.

2.4.2.7 Methyl red- Vogues Proskauer (MRVP) test:

The MRVP medium (*Appendix B*) test tubes have been inoculated with the isolated strains. The tubes have been incubated at 30°C for 48 h. 2.5 ml of broth taken and placed in sterile test tube which has been further used for Vogues Proskauer test and then 3-4 drops of MR indicator added into original broth. A distinct red color indicates the positive test; yellow color indicates a negative test. To earlier taken 2.5 ml MRVP broth, 0.6 ml reagent VP A (napthol reagent) and 0.2 ml reagent VP B (*Appendix B*) has been added. Reaction observed for 30 minutes. For positive reaction medium color changes to pink or red color while for negative reaction no color change or color changes to copper color.

2.4.2.8 Citrate test:

Citrate agar test (*Appendix B*) tubes have been inoculated with the isolated strains.
The inoculated test tubes have been incubated at 30°C for 24 h. A positive result has color change of the media from green to blue while negative result has no color change.

2.4.2.9 Carbohydrate fermentation test:

Phenol red carbohydrate broth (*Appendix B*) medium have been prepared in test tubes**.** Glucose, fructose and lactose have been tested for fermentation. Single carbohydrate used for each batch of medium. Test tubes have been inoculated with the isolated strains. The inoculated test tubes have been incubated at 30°C for 24 h. A yellow color indicates that enough acid products have been produced by fermentation of the sugar to lower the pH to 6.8 or less. A reddish or pink color indicates a negative reaction.

2.4.2.10 Urease test:

Christensen's Urea agar (*Appendix B*) test tubes have been inoculated with isolated strains. The inoculated test tubes have been incubated at 30°C and the slants have been observed for a color change at 6 hours, 24 hours, and every day for up to 6 days. A positive result has color change of the media from yellow to pink. The culture medium will remain a yellowish color if the organism is urease negative.

2.4.3 Scanning Electron Microscope:

For every bacterial sample to be prepared for SEM, it is essential to fix them first in order to preserve their structure (Kalab et al., 2008). The overnight grown cultures of strain PS3 and strain OS1 have been individually centrifuged at 8000 rpm for 10 minutes. The pellet has been washed twice with phosphate buffer (pH 7.4) and subsequently fixation done in 2% glutaraldehyde for period of one hour (Glauert, 1975). Fixed specimens have been washed with the phosphate buffer (pH 7.4) and dehydrated in ethanol series i.e. 30,50,75,90 and 100% ethanol. The Scanning Electron Microscopy has been performed using JEOL (JSM, Japan) Scanning Electron Microscopy attached to an EDX unit, with magnification from 10X up to 400,000X and resolution 3.5 nm.

2.4.4 Sequencing of 16S rDNA and Phylogenetic analysis:

The 16s rDNA based molecular technique has been performed for the isolated strain PS3 and strain OS1 at Xcelris genomics, Ahmedabad, Gujarat, India. The step wise experimental procedure for 16s rDNA molecular technique is described below:

2.4.4.1 Extraction of Genomic DNA from pure Culture:

The bacterial sample has been spun till desired amount of pellet has been obtained (1ml of culture corresponding to 1 O.D).It has been washed twice with distill water. The pellet was resuspended in 567µl of TE buffer (*Appendix-B*) by repeated pippetting and vortexing. In this bacterial suspension 30µl of 10% SDS and 15µl of RNase (10 mg/ml)

and Proteinase K (20 mg/ml) have been incubated at 37^0C for 30 minutes. 80µl CTAB-NaCl (*Appendix-B*) solution has been added to the above mixture and was mixed thoroughly. It has been incubated for 10 minutes at 65ºC. The addition of RNase (30 µg of RNase /ml) has been also added initially to remove RNA contamination.

Phenol Chloroform Treatment:

- About 250µl of Tris saturated phenol (*Appendix-B*) and 250µl chloroform has been added to the tube after incubation.
- It has been thoroughly mixed by inverting the tube carefully.
- The tube was spanned at 12,000 rpm for 10 minute and the upper layer has been transferred to a fresh tube.
- Equal volume of chloroform has been added and rocked for 15 minutes.
- The tube was centrifuged at 12,000rpm for 10 minutes and the supernatant has been transferred to a fresh tube.
- $1/10^{th}$ volume of 3M-sodium acetate of pH 5.2 and 0.7 volume of Isopropanol has been added and incubated at room temp for overnight.
- After incubation it has been centrifuged at 14,000 rpm for 30 minutes and supernatant has been discarded.
- The pellet has been washed with 70% ethanol.
- The pellet has been air dried completely and dissolved in 50µl double distilled water.

2.4.4.2 Quantitation and Quality Assessment of DNA:

The DNA stock samples has been quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultra violet (UV) absorbance has been checked at 260 and 280 nm for determination of DNA concentration and purity. Purity of DNA has been judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 has been considered to be of good purity.

Concentration of DNA has been estimated using the following formula:

Concentration of DNA (mg/ml) = Optical Density at 260nm x 50 x Dilution factor

Quality and purity of DNA have been checked by agarose gel electrophoresis. 0.8% Agarose (w/v) in 0.5X TAE (pH 8.0) buffer (Sambrook and Russell, 2001) (*Appendix B*) has been used for submarine gel electrophoresis. Ethidium bromide (1%) has been added at 10µl /100ml. The wells have been charged with 5µl of DNA preparations mixed with

1µl gel loading dye. Electrophoresis has been carried out at 80V for 30 min at room temperature. DNA has been visualized under UV using UV transilluminator. The DNA has been used further for PCR.

2.4.4.3 Amplification 16S rRNA gene:

16S rRNA gene fragment has been amplified by PCR from genomic DNA using 16S rRNA gene universal primers:

8F: 5'AGAGTTTGATCCTGGCTCAG3'

1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'

PCR has been carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube in Eppendorf Thermal Cycler. Composition of reaction mixture for PCR is given in Table 2.1.

Table 2.1: Composition of reaction mixture for PCR

Components	**Quantity**	**Final Concentration**
DNase-RNase free water	7.50 µl	--
2X PCR master mix (MBI Fermentas)	12.50 µl	1X
Forward Primer (10 pmole/µl)	1.00 µl	10 pmole
Reverse Primer (10 pmole/µl)	1.00 µl	10 pmole
Diluted DNA (30ng/µl)	3.0 µl	--
Total	**25.00 µl**	--

PCR tubes containing the mixture have been tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components have been transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given in Table 2.2.

Table 2.2: Steps and conditions of thermal cycling for PCR

Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95°C	2 min	1
Final Denaturation	94°C	30 s	30
Annealing	52°C	30 s	
Extension	72°C	90 s	
Final Extension	72°C	10 min	1

2.4.4.4 Visualization of PCR Product:

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube has been mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % agarose gel

containing ethidium bromide (1 per cent solution at 10 µl/100 ml) at constant 5V/cm for 30 min in 0.5 X TAE buffer. The amplified product has been visualized as a single compact band of expected size under UV light and documented by gel documentation system.

2.4.4.5 Purification of PCR product:

Amplified PCR product has been purified using Qiagen Mini elute Gel extraction kit according to the manufacture's protocol.

2.4.4.6 Sequencing of Purified 16S rRNA Gene Segment:

The concentration of the purified 16S rRNA Gene Segment has been determined and has been subjected to automated sequencing on ABI 3730xl Genetic Analyzer. Cycle sequencing has been performed following the instructions supplied along with BigDye® Terminator v3.1 Cycle Sequencing Kit. The reaction has been carried out in a final reaction volume of 20µl using 200µl capacity thin wall PCR tube. The cycling protocol (Table 2.3) has been designed for 25 cycles with the thermal ramp rate of 1°C per second.

Table 2.3: Cycling protocol for sequencing reaction

Step	Temperature	Time
Denaturation	96°C	10 s
Annealing	52°C	5 s
Extension	60°C	4 min

The three steps have been repeated for 25 cycles. After cycling, the extension products have been purified and mixed well in 10 µl of Hi-Di formamide. The contents have been mixed on shaker for 30 minutes at 300 x g. Eluted PCR products have been placed in a sample plate and covered with the septa. Sample plate has been heated at 95°C for 5 min, snap chilled and loaded into auto sampler of the instrument.

2.4.4.7 Electrophoresis and Data Analysis:

Electrophoresis and data analysis has been carried out on the ABI 3730xl Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matrix files.

2.4.4.8. Sequence Analysis:

Both ends of the sequence have been verified with the chromatogram file. The sequence has been converted into fasta format and saved in notepad. The 16S rRNA gene sequence has been used to carry out BLAST (Basic local Alignment Search tool) with nr database of

NCBI (National Centre for Biotechnology Information) Genbank using MEGABLAST algorithm. The BLAST data has been arranged in maximum percentage identity and first ten sequences has been selected and exported in FASTA format. Based on maximum identity score and query coverage the best highly identical 10 sequences have been selected and aligned using multiple alignment software program ClustalW (MEGA4 tool). The evolutionary history has been inferred using the Neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates has been taken to represent evolutionary history of the taxa analyzed. The evolutionary distances have been computed using the Kimura 2- parameters method. Phylogenetic analysis has been conducted in MEGA software version 4 (Tamura et al., 2007).

2.5 Analytical methods

2.5.1 Estimation of biomass:

For measuring biomass, the 1 ml of overnight cultures has been centrifuged at 8000 rpm for 10 minutes. The pellet has been taken for estimation of biomass and it has been resuspended in distilled water. This suspension has been measured against distilled water as reference at 600 nm using UV/Visible spectrophotometer. The calibration curve of biomass for both the strains is given in *Appendix- A*.

2.5.2 Estimation of phenol:

Direct photometric method (APHA, 1998) has been used for determination of phenol in medium. The samples have been centrifuged at 8000 rpm for 10 minutes. The supernatant has been used for phenol estimation. In the Direct photometric method, Phenolic material reacts with 4-aminoantipyrine in the presence of potassium ferricyanide at a pH 7.9 ± 0.1 to form a stable reddish-brown antipyrene dye with maximum absorbance at 500 nm. The amount of color produced is a function of the concentration of phenolic material and estimation of phenol has been done by using UV/Visible spectrophotometer. The calibration curve of phenol and detailed procedure of estimation are given in *Appendix-A* and *C* respectively.

2.6 Inoculum development

Fresh culture of bacterium grown on nutrient agar slants has been inoculated in nutrient broth and incubated at 30°C and 150 rpm for 24 h. These cells have been subsequently used as inoculum for optimization and degradation experiments.

2.7 Optimization of medium components and physiological conditions for phenol degradation

Optimization of medium components and physiological conditions is of primary importance in biodegradation processes. Parameters like initial phenol concentration, pH, temperature, inoculum size and media components have effect on phenol degradation (Bandyopadhyay et al., 1998; Annadurai et al., 2008; Balamurugan et al., 2012). Hence, in present study pH, temperature, phenol, inoculum size, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NaCl and $MgSO_4$ have been considered for optimization of phenol degradation.

The one factor at a time approach is laborious, time-consuming and less capable to find true optimum levels due to the interactions among variables (Tang et al., 2004). On the other hand, statistical planned experiments effectively solve such problems and minimize the error in determining the effect of parameters (Lakshmi et al., 2011). Statistical experimental designs are used for optimization strategies like screening experiments and determination of optimum conditions for targeted responses (Abdel-Fattah et al., 2005). Statistical experimental designs such as Plackett-Burman and central composite design (CCD) have been successfully used to optimize many bioprocesses (Gaur et al., 2008; Reddy et al., 2008; Zhou et al., 2011; Sivasubramanian and Namasivayam, 2014). Hence to overcome the drawbacks of one factor at a time approach, statistically designed experiments have been employed for optimization of phenol degradation by isolated strains PS3 and OS1 independently.

In order to optimize phenol degradation by strain PS3 and OS1 using statistically designed experiments, the levels (range) of parameters (variables) has to be known. Hence preliminary experiments have been performed to determine the levels of pH, temperature, phenol concentration and inoculum size. In the preliminary experiments, these factors (parameters) have been studied as a single variable (One factor at a time method). The effect of pH (6, 7, 8 and 9), temperature (25, 30, 35 and 40°C), phenol (100, 150, 200, 250, 300, 350 and 400 mg/l) and inoculum size (3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 %, (v/v)) has been investigated for both the strains independently. The levels of KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NaCl and $MgSO_4$ have been fixed as per media composition mentioned in section 2.3.2. In the next step, screening has been done by using Plackett- Burman design to obtain significant factors and further the optimum levels of significant factors have been found by using central composite design.

Statistical software Design Expert (Trial version) (Stat-Ease Inc., Minneapolis, USA) has been used for designing experiments, regression analysis and to plot the response surface graphs. All degradation experiments have been performed in 250 ml Erlenmeyer flaks containing 100 ml degradation mineral salt media and incubated at 150 rpm for 30 h and 36 h for strain PS3 and OS1 respectively. The determination of biomass and phenol concentration in sample has been done as per procedure mentioned in section 2.5. All experiments have been performed in triplicates and the average of the independent experiments has been produced as the result.

2.7.1 Screening of significant factors by using Plackett-Burman design:

Plackett Burman design allows screening of main factors from large number of variables (Reddy et al., 2008). Nine important parameters (variables) for phenol degradation have been selected for screening via Plackett-Burman design and each variable has been studied at three levels with respect to their main effect on phenol degradation.

The Plackett Burman design is based on the first order polynomial model:

$$\boldsymbol{Y = \beta_0 + \sum \beta_i X_i} \qquad (\mathbf{2.1})$$

where, Y denotes the response (phenol degradation in %), β_0 is model intercept, β_i is factor linear coefficient and X_i is the level of the independent factor. The details of eleven variables (including two dummy variables) and their levels selected for independent phenol degradation by strains PS3 and OS1 are shown in Table 2.4 and 2.5 respectively.

Table 2.4: Eleven variables and their levels used in Plackett-Burman design for strain PS3

Variable code	Variables	Lower level (-1)	Center level (0)	Higher level (+1)
X_1	pH	6	7	8
X_2	Temperature ($^{\circ}$C)	25	30	35
X_3	Phenol (mg/l)	200	300	400
X_4	Inoculum size (%, v/v)	2	5	8
X_5	KH_2PO_4 (mg/l)	100	200	300
X_6	K_2HPO_4 (mg/l)	300	400	500
X_7	$(NH_4)_2SO_4$ (mg/l)	300	400	500
X_8	NaCl (mg/l)	50	100	150
X_9	$MgSO_4$ (mg/l)	50	100	150
X_{10}	Dummy 1	-1	0	+1
X_{11}	Dummy 2	-1	0	+1

Table 2.5: Eleven variables and their levels used in Plackett-Burman design for strain OS1

Variable code	Variables	Lower level (-1)	Center level (0)	Higher level (+1)
X_1	pH	6	7	8
X_2	Temperature ($^{\circ}$C)	25	30	35
X_3	Phenol (mg/l)	150	250	350
X_4	Inoculum size (%, v/v)	3.5	6.5	9.5
X_5	KH_2PO_4 (mg/l)	100	200	300
X_6	K_2HPO_4 (mg/l)	300	400	500
X_7	$(NH_4)_2SO_4$ (mg/l)	300	400	500
X_8	NaCl (mg/l)	50	100	150
X_9	$MgSO_4$ (mg/l)	50	100	150
X_{10}	Dummy 1	-1	0	+1
X_{11}	Dummy 2	-1	0	+1

Table 2.6: Plackett-Burman experimental design for strain PS3

pH	**Temperature** ($^{\circ}$C)	**Phenol** (mg/l)	**Inoculum size** (%, v/v)	**KH_2PO_4** (mg/l)	**K_2HPO_4** (mg/l)	**$(NH_4)_2SO_4$** (mg/l)	**NaCl** (mg/l)	**$MgSO_4$** (mg/l)	**Dummy 1**	**Dummy 2**
8	35	200	8	300	500	300	50	50	1	-1
6	35	400	2	300	500	500	50	50	-1	1
8	25	400	8	100	500	500	150	50	-1	-1
6	35	200	8	300	300	500	150	150	-1	-1
6	25	400	2	300	500	300	150	150	1	-1
6	25	200	8	100	500	500	50	150	1	1
8	25	200	2	300	300	500	150	50	1	1
8	35	200	2	100	500	300	150	150	-1	1
8	35	400	2	100	300	500	50	150	1	-1
6	35	400	8	100	300	300	150	50	1	1
8	25	400	8	300	300	300	50	150	-1	1
6	25	200	2	100	300	300	50	50	-1	-1
7	30	300	5	200	400	400	100	100	0	0
7	30	300	5	200	400	400	100	100	0	0
7	30	300	5	200	400	400	100	100	0	0
7	30	300	5	200	400	400	100	100	0	0

Table 2.7: Plackett-Burman experimental design for strain OS1

pH	**Temperature** ($^{\circ}$C)	**Phenol** (mg/l)	**Inoculum size** (%, v/v)	**KH_2PO_4** (mg/l)	**K_2HPO_4** (mg/l)	**$(NH_4)_2SO_4$** (mg/l)	**NaCl** (mg/l)	**$MgSO_4$** (mg/l)	**Dummy 1**	**Dummy 2**
8	35	150	9.5	300	500	300	50	50	1	-1
6	35	350	3.5	300	500	500	50	50	-1	1
8	25	350	9.5	100	500	500	150	50	-1	-1
6	35	150	9.5	300	300	500	150	150	-1	-1
6	25	350	3.5	300	500	300	150	150	1	-1

6	25	150	9.5	100	500	500	50	150	1	1
8	25	150	3.5	300	300	500	150	50	1	1
8	35	150	3.5	100	500	300	150	150	-1	1
8	35	350	3.5	100	300	500	50	150	1	-1
6	35	350	9.5	100	300	300	150	50	1	1
8	25	350	9.5	300	300	300	50	150	-1	1
6	25	150	3.5	100	300	300	50	50	-1	-1
7	30	250	6.5	200	400	400	100	100	0	0
7	30	250	6.5	200	400	400	100	100	0	0
7	30	250	6.5	200	400	400	100	100	0	0
7	30	250	6.5	200	400	400	100	100	0	0

The 16 runs with four centre points have been performed individually for isolated strains PS3 and OS1 and on the basis of response the regression coefficient values have been generated. Plackett-Burman experimental design runs for eleven variables are given in Table 2.6 and 2.7 for strain PS3 and strain OS1 respectively. The isolated strains PS3 and OS1 have been individually inoculated in mineral salt media where nutrient concentrations and physical conditions have been maintained as per experimental runs. From regression analysis, the factors showing *p*-values below 0.05 have been considered to have significant effect on phenol degradation and used further for central composite design (CCD).

2.7.2 Optimization of significant factors by using Response Surface Methodology:

The statistical experiment designs most widely used in optimization experiments are termed response surface designs. The application of central composite design (CCD) under response surface methodology (RSM) assisted in both modeling and optimization of medium components and growth conditions (Box and Wilson, 1951). By using Plackett Burman design it has been found that pH, temperature, phenol concentration and inoculum size have significant effect on phenol degradation by strain PS3. For strain OS1, it has been found that pH, temperature, phenol concentration, inoculum size and $(NH_4)_2SO_4$ concentration have significant effect on phenol degradation. The full factorial central composite design has been used to study effect of these independent variables on phenol degradation by strains PS3 and OS1 individually. Each of the independent variables has been studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$), where $\alpha = 2$. The details of variables and their levels studied for each isolated strain is given in Table 2.8 and 2.9.

Table 2.8: Four variables and their levels used in central composite design for strain PS3

	Coded level				
Variables	**- 2**	**-1**	**0**	**+1**	**+ 2**
pH	6	6.5	7	7.5	8
Temperature (°C)	26	28	30	32	34
Phenol (mg/l)	200	250	300	350	400
Inoculum size (%, v/v)	2	3.5	5	6.5	8

Table 2.9: Five variables and their levels used in central composite design for strain OS1

	Coded level				
Variables	**- 2**	**-1**	**0**	**+1**	**+ 2**
pH	6	6.5	7	7.5	8
Temperature (°C)	26	28	30	32	34
Phenol (mg/l)	150	200	250	300	350
Inoculum size (%, v/v)	3.5	5	6.5	8	9.5
$(NH_4)_2SO_4$ (mg/l)	300	350	400	450	500

A full factorial central composite design has been used with 30 experiments for isolated strain PS3 and these 30 experiments having 2^4 = 16 cube points, 6 centre points and 8 axial points. For strain OS1, a full factorial central composite design has been used with 50 experiments and these 50 experiments having 2^5 = 32 cube points, 8 centre points and 10 axial points.

The second-order polynomial equation has been used to calculate the relationship between the independent variables and the response is as follows:

$$\boldsymbol{Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j} \qquad (\mathbf{2.2})$$

where, Y denotes the response (% degradation of phenol), X_i and X_j are input variables, β_0 is model intercept, β_i is factor estimates, β_{ii} is the i^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient. The details of experimental design are given in Table 2.10 and 2.11 for isolated strain PS3 and OS1 respectively. The isolated strains PS3 and OS1 have been individually inoculated in mineral salt media containing nutrient concentrations and physiological conditions as per runs mentioned in Table 2.10 and 2.11 respectively. The other nutrients have been kept at their average values (centre values) throughout the study.

Table 2.10: Experimental set up for isolated strain PS3 as per full factorial Central Composite design

Run	**pH**	**Temperature** (°C)	**Phenol** (mg/l)	**Inoculum size** (% v/v)
1	7.5	32	250	6.5
2	6.5	32	250	3.5
3	7.5	28	250	6.5
4	6.5	28	250	3.5
5	7	30	300	5
6	7.5	32	250	3.5
7	7.5	28	350	3.5
8	7	34	300	5
9	7	30	300	5
10	6	30	300	5
11	7	30	300	8
12	6.5	32	250	6.5
13	7.5	32	350	3.5
14	8	30	300	5
15	7	30	300	5
16	7	30	300	5
17	6.5	28	350	6.5
18	7.5	28	350	6.5
19	7	26	300	5
20	7.5	28	250	3.5
21	7	30	300	5
22	7.5	32	350	6.5
23	6.5	28	350	3.5
24	7	30	300	2
25	6.5	32	350	3.5
26	7	30	400	5
27	6.5	28	250	6.5
28	6.5	32	350	6.5
29	7	30	200	5
30	7	30	300	5

Table 2.11: Experimental set up for isolated strain OS1 as per full factorial Central Composite design

Run	**pH**	**Temperature** (°C)	**Phenol** (mg/l)	**Inoculum size** (%, v/v)	**$(NH_4)_2SO_4$** (mg/l)
1	6.5	32	300	5	450
2	6.5	28	300	8	350
3	7	34	250	6.5	400
4	7.5	32	200	5	450
5	7.5	28	300	5	350
6	7.5	32	200	8	450
7	7.5	28	200	8	450
8	7.5	32	200	5	350

9	7	30	250	6.5	400
10	6.5	32	300	8	450
11	7	30	150	6.5	400
12	6.5	32	200	8	350
13	7	30	250	6.5	400
14	7	30	250	6.5	400
15	7	30	350	6.5	400
16	7	30	250	6.5	400
17	7	30	250	3.5	400
18	7.5	32	300	5	350
19	7	26	250	6.5	400
20	7.5	28	300	5	450
21	6.5	28	300	5	350
22	6.5	28	300	5	450
23	7.5	28	300	8	450
24	6	30	250	6.5	400
25	7	30	250	6.5	400
26	7.5	28	300	8	350
27	6.5	28	200	5	450
28	7.5	32	300	5	450
29	7.5	28	200	5	450
30	7	30	250	6.5	500
31	6.5	28	300	8	450
32	7.5	32	200	8	350
33	7.5	32	300	8	450
34	7.5	28	200	5	350
35	8	30	250	6.5	400
36	7	30	250	6.5	400
37	7.5	32	300	8	350
38	6.5	28	200	5	350
39	6.5	32	200	5	450
40	6.5	32	200	8	450
41	6.5	28	200	8	350
42	6.5	32	200	5	350
43	7	30	250	6.5	300
44	7	30	250	9.5	400
45	6.5	32	300	8	350
46	7	30	250	6.5	400
47	6.5	28	200	8	450
48	7.5	28	200	8	350
49	7	30	250	6.5	400
50	6.5	32	300	5	350

2.8 Experimental Validation of the predicted model

From statistically designed experiments, the predicted levels have been obtained for the significant factors. To validate these results, the independent experiments have been performed at predicted levels of these factors for strain PS3 and strain OS1 in 250 ml Erlenmeyer flasks containing 100 ml degradation mineral salt media and incubated at 150 rpm for 30 h and 36 h respectively. The determination of biomass and phenol concentration in sample has been done as per procedure mentioned in section 2.5. Each experiment has been done in triplicate under the same operating conditions and average values of residual phenol concentrations of three independent experiments have been reported.

2.9 Study of biodegradation of phenol

Phenol degradation study by isolated strain PS3 and strain OS1 has been done at their respective obtained optimum levels of parameters various initial phenol concentrations. For each experiment freshly prepared inoculum has been used and the experiments have been performed in 250 ml Erlenmeyer flask containing 100 ml mineral salt medium with various initial phenol concentrations in batch mode and at 150 rpm (Fig.2.1). Phenol degradation ability of strain PS3 and strain OS1 has been studied by culturing in mineral salt medium with various initial phenol concentrations.

Fig.2.1. Experimental set up for degradation study of phenol by isolated strains

Each experiment has been performed until the residual concentration of phenol in flask has been found to saturate with time. Each experiment has been done in triplicate under the same operating conditions and average values of residual phenol concentrations of

three independent experiments have been reported. The reaction mixture containing all media components except bacterial inoculums have been used as control.

2.10 Growth Kinetics of isolated strains for phenol biodegradation

The relationship between the specific growth rate, μ and substrate concentration, S must be quantified to design and operate effective biological toxic waste treatment. Evaluation of the biokinetic constants is significant for understanding the capacities of the microorganisms for the degradation and for the design and operation of biological reactors. The various substrate utilization and inhibitory models have been extensively studied for growth kinetics of microbes on phenol (Sahoo et al, 2011; Dey and Mukherjee, 2010). Haldane model is widely accepted for representing growth kinetics of microbes for inhibitory compounds like phenol due to its mathematical simplicity and accuracy (Kumar et al., 2005, Rigo and Alegre, 2004). Agarry et al. (2010) also have reported that the Haldane model proposed as one of the best models to describe the phenol degradation behavior. Hence, Haldane model has been used for growth kinetic analysis for phenol degradation by isolated strains.

Haldane model equation relates microbial specific growth rate (μ) and limiting substrate concentration (S) as follows:

$$\boldsymbol{\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_i}}} \qquad (\mathbf{2.3})$$

where, μ_{max} is maximum specific growth rate (h^{-1}), K_s is half-saturation coefficient (mg/l) and K_i is the substrate inhibition constant (mg/l). Specific growth rate of the culture at different substrate concentrations in batch system has been calculated as per the following relationship:

$$\boldsymbol{\mu = \frac{1}{X}\frac{dX}{dt}} \qquad (\mathbf{2.4})$$

where μ is specific growth rate (h^{-1}), X is biomass concentration (mg/l) and t is time (h). The growth kinetic parameters μ_{max}, K_i and K_s for the strains PS3 and OS1 have been estimated by fitting their respective experimental growth data at phenol concentration range 0 -1500 mg/l and 0 – 1250 mg/l respectively to Haldane Kinetic Model. This model has been solved by the use of a non-linear regression method using MATLAB V 7.11.

2.11 Immobilization of isolated strains

To enhance phenol degradation efficiency of the strains PS3 and OS1, the cells have been immobilized on calcium alginate beads and this method is known as entrapment method. Subsequently, degradation study has been done for different phenol concentrations by immobilized cells. For degradation experiments, pH and temperature have been maintained at optimum values obtained for free cells.

2.11.1 Production of inoculum for preparation of immobilized cells:

Strain PS3 and strain OS1 have been inoculated in sterile nutrient broth and have been incubated for 24 h at 30°C, 150 rpm in 250 ml Erlenmeyer flasks independently. The cells obtained from nutrient broth have been used for immobilization procedure.

2.11.2 Production of immobilized cells:

Liquid cultures have been centrifuged 10000 rpm at 4^{o}C for 10 min and the supernatant has been discarded. The pellet (10 g wet weight approx.) has been resuspended in 10 ml phosphate buffered saline (PBS). Bacterial cell suspension (20 ml) has been added to 100 ml previously autoclaved 3% (w/v) sodium alginate solution and then bacterial culture - sodium alginate mixture has been dropped from height of about 20 cm through syringe with needle into 3% (w/v) Calcium chloride solution (Park et al., 2013). The beads have been formed immediately (Fig.2.2). After immobilization, the beads have been incubated at room temperature in the Calcium chloride solution for 2h and then stored in this solution overnight at 4°C (Bandhyopadhyay et al., 2001).

Fig.2.2. Cells immobilized in Calcium alginate beads

2.12 Degradation study of phenol by immobilized cells

Phenol degradation ability of immobilized strain PS3 and strain OS1 has been studied by culturing in 100 ml mineral salt medium with various phenol concentrations. For strain OS1, optimum concentration of $(NH_4)_2SO_4$ derived for free cells has been used. The flasks have been incubated in batch mode at 150 rpm. Each experiment has been performed until the residual concentration of phenol in flask has been found to saturate with time. Each experiment has been done in triplicate under the same operating conditions and average values of residual phenol concentrations of three independent experiments have been reported.

RESULTS AND DISCUSSION

Chapter-3

Results and Discussion

Industrial phenolic effluent requires proper treatment before being discharged into the environment. Biological method is the attractive method of treatment of phenolic effluent as it is economical, practical and the most promising and versatile approach as it leads to complete mineralization of phenol producing non toxic end products. Contaminated soil often makes adaptation of different metabolic pathways and hence creates high biodiversity in soil microorganisms (Alloway, 2001). Hence, contaminated soil is always a good source in order to isolate phenol degrading strains. In spite of the toxicity of phenol, a number of microorganisms have been reported to degrade phenol.

In present investigation, phenol degrading bacterial strains have been isolated from soil contaminated with paper mill effluent and crude oil. The most prominent phenol degrading strains have been identified and characterized. The detailed optimization of medium components and physiological conditions has been studied for phenol degradation as a response. The ability of strains to grow at high phenol concentrations has been also studied. This work also aims to study phenol biodegradation by immobilized cells of isolated strains.

3.1 Isolation of phenol degrading strains from contaminated soil

Soil samples have been collected from paper mill treated effluent discharge site and crude oil spillage site of oil refinery. Soil samples have been enriched in mineral salt media containing phenol as sole source of carbon. After enrichment, twenty five and twenty strains have been obtained from soil sample collected from paper mill and oil refinery site respectively. The strains obtained after enrichment have been further treated with increasing phenol concentrations to get high phenol concentration tolerant strains of bacteria. It has been found that strain PS3 tolerates up to 1500 mg/l phenol among strains obtained from paper mill site while strain OS1 tolerates up to 1250 mg/l phenol among strains obtained from oil refinery site. Since these strains shown promising degradation efficiency, they have been considered as the subject of the current study.

3.2 Identification of isolated phenol degrading strains

Identification of strains PS3 and OS1 has been done on the basis of their morphological, biochemical characteristics and molecular characteristics.

3.2.1 Morphological and Biochemical Characteristics

Morphological characteristics of isolated strains have been observed by spread plate technique. Biochemical tests have been performed for both the isolated strains independently. Fig.3.1 and 3.2 shows the colony morphology of the isolated strains PS3 and OS1 respectively. Morphological and biochemical characteristics of isolates are enlisted in Table 3.1.

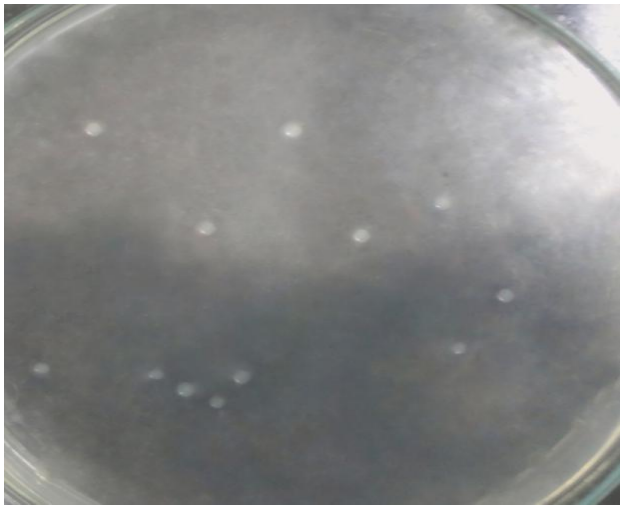


Fig.3.1. Colonies of strain PS3

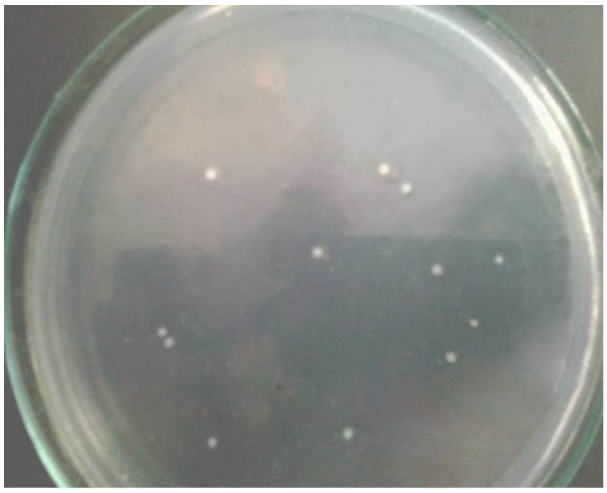


Fig.3.2. Colonies of strain OS1

3.2.2 SEM analysis of isolated strains

The microorganism has been fixed to glass slides with the help of glutaraldehyde fixation method under subsequent drying with increasing concentration of ethanol. The magnification of the microscope has been 5000 X for strain PS3 and 3500 X for strain

OS1. The isolated strain PS3 and strain OS1 have been found to be coccobacillus and rod shaped bacillus as shown in Fig. 3.3 and Fig. 3.4 respectively.

Table 3.1: Morphological and Biochemical characteristics of strain PS3 and strain OS1

Characteristics	**Strain PS3**	**Strain OS1**
Size	1-2 mm	1-2 mm
Shape	Circular	Irregular
Colour	Grey	Slight yellowish
Margin	Entire	Undulate
Opacity	Opaque	Opaque
Elevation	Convex	Flat
Textures	Viscous	Viscous
Grams nature	Gram negative	Gram positive
Motility	Motile	Motile
Oxygen requirement	Aerobic	Aerobic
Catalase test	+	+
Oxidase test	+	+
Nitrate reduction	-	-
Indole test	-	-
Glucose fermentation	+	+
Fructose fermentation	+	+
Lactose fermentation	+	-
Urease test	-	-
Citrate test	+	+
Gelatin liquefaction	+	-
Starch hydrolysis	-	-
Methyl red test	-	+
Vogues Proskauer test	-	+

+: Positive reaction; - : Negative reaction

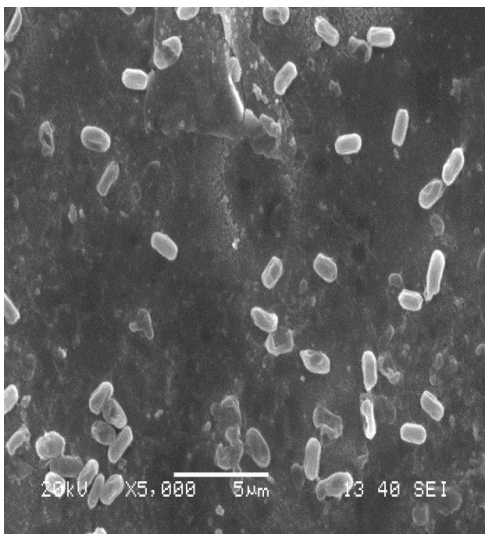


Fig.3.3. SEM image of strain PS3

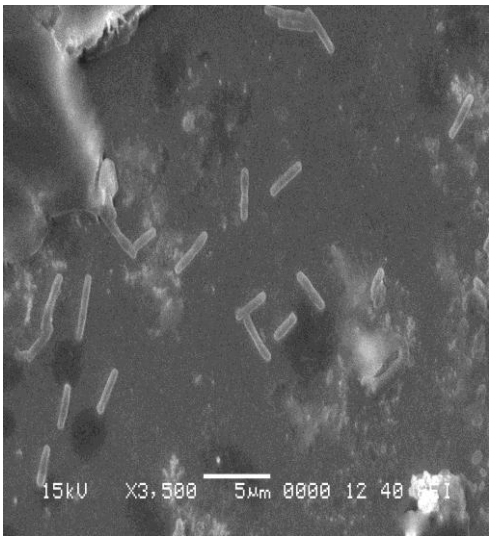
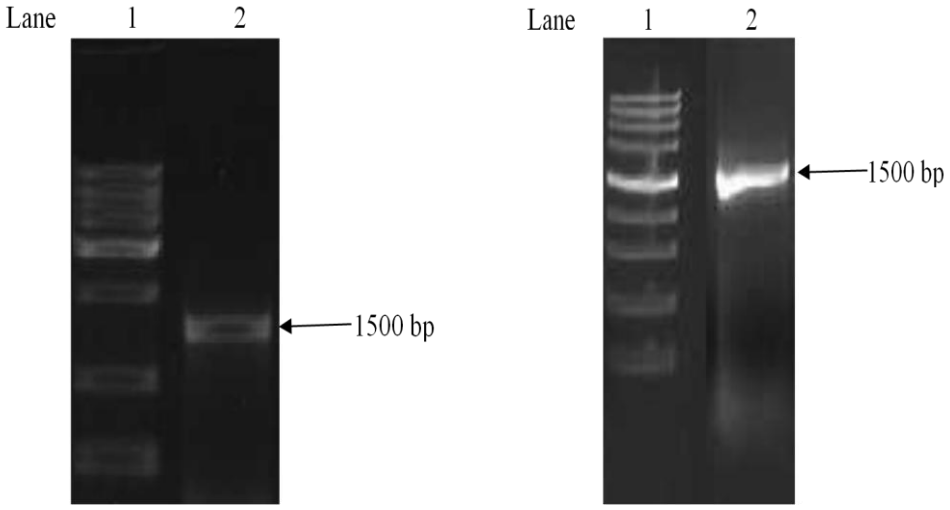


Fig.3.4. SEM image of strain OS1

3.2.3 Molecular characterization

DNA has been isolated independently from the overnight culture of the isolated strain

PS3 and strain OS1. A single band of high-molecular weight DNA has been observed on 1.2% Agarose Gel. Fragment of 16S rRNA gene has been amplified by PCR and a single discrete PCR amplicon band of 1500 bp has been observed when resolved on Agarose Gel (Fig. 3.5).



(A) (B)

Fig.3.5. Gel Image of 16S rDNA amplicon of (A) strain PS3 and (B) strain OS1.
(Lane 1: DNA marker; Lane 2: 16S rDNA amplicon band).

Forward and reverse DNA sequencing reaction of PCR amplicon has been carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1311bp 16S rRNA gene of strain PS3 and consensus sequence of 1282bp 16S rRNA gene of strain OS1 have been generated from forward and reverse sequence data using aligner software BioEdit.

3.2.3.1 Distance Matrix and phylogenetic tree:

The obtained 16S rRNA gene sequences have been used to carry out BLAST with the nr GenBank database (non-redundant database) of NCBI. Based on maximum identity score first ten sequences have been selected and aligned using multiple sequence alignment software program ClustalW. The sequence producing significant alignments for the isolated strain PS3 and OS1 are shown in Table 3.2 and 3.3 respectively.

Ribosomal Database Project (RDP) provides data, tools and services related to ribosomal RNA sequences. Distance matrix has been generated using RDP database (Table 3.4 and 3.5). Distance matrices have been applied to phenetic data using a matrix of pairwise distances. These distances have been then reconciled to produce a tree (a phylogram,

with informative branch lengths) and the Phylogenetic tree has been constructed using MEGA software version 4 (Fig. 3.6 and 3.7).

Table 3.2: Sequence producing significant alignments for strain PS3

Accession	Description	Max. score	Total score	Query coverage	E value	Max. identity
KF536883.1	*Burkholderia* sp. FCD2-1	2416	2416	100%	0.0	99%
KC833503.1	*Burkholderia* sp. TCP10	2416	2416	100%	0.0	99%
KC462881.1	*Burkholderia* sp. T	2416	2416	100%	0.0	99%
HE821232.1	*Burkholderia* sp. WK11	2416	2416	100%	0.0	99%
HE821231.1	*Burkholderia* sp. WK10	2416	2416	100%	0.0	99%
JN872503.1	*Burkholderia* sp. SAP27_1	2416	2416	100%	0.0	99%
JN622010.1	*Burkholderia* sp. WN-2	2416	2416	100%	0.0	99%
HQ231941.1	*Burkholderia* sp. EW7	2416	2416	100%	0.0	99%
GQ383907.1	*Burkholderia cepacia* strain 2EJ5	2416	2416	100%	0.0	99%
FJ823011.1	*Burkholderia* sp. gx-152	2416	2416	100%	0.0	99%

Table 3.3: Sequence producing significant alignments for strain OS1

Accession	Description	Max. score	Total score	Query coverage	E value	Max. identity
KF059271.1	*Bacillus* sp. yj-1	2362	2362	99%	0.0	100%
KF059268.1	*Bacillus* sp. hg-4	2362	2362	99%	0.0	100%
KF562256.1	*Bacillus* sp. IHB B 3460	2362	2362	99%	0.0	100%
KF535137.1	*Bacillus pumilus* strain BAB-1846	2362	2362	99%	0.0	100%
KF535136.1	*Bacillus pumilus* strain BAB-1845	2362	2362	99%	0.0	100%
KF535135.1	*Bacillus pumilus* strain BAB-1844	2362	2362	99%	0.0	100%
KF535132.1	*Bacillus pumilus* strain BAB-2837	2362	2362	99%	0.0	100%
KF535124.1	*Bacillus pumilus* strain BAB-1320	2362	2362	99%	0.0	100%
KF535123.1	*Bacillus pumilus* strain BAB-1315	2362	2362	99%	0.0	100%
KF307204.1	*Bacillus* sp. HYJY	2362	2362	99%	0.0	100%

The evolutionary history has been inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances have been computed using the Kimura 2-parameter method

(Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included have been 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data have been eliminated from the dataset (Complete deletion option).

Table 3.4: Distance Matrix for strain PS3

Strain PS3	1		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
KF536883.1	2	0.001		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KC833503.1	3	0.001	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KC462881.1	4	0.001	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
HE821232.1	5	0.001	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
HE821231.1	6	0.001	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
JN872503.1	7	0.001	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
JN622010.1	8	0.001	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
HQ231941.1	9	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
GQ383907.1	10	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
FJ823011.1	11	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

Table 3.5: Distance Matrix for strain OS1

Strain OS1	1		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KF059271.1	2	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KF059268.1	3	0.000	0.000		0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
KF562256.1	4	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
KF535137.1	5	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
KF535136.1	6	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
KF535135.1	7	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
KF535132.1	8	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
KF535124.1	9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
KF535123.1	10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
KF307204.1	11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

Based on morphological, biochemical characteristics, nucleotide homology and phylogenetic analysis, the isolated strain PS3 belongs to the genus *Burkholderia* and the strain OS1 has been found to be *Bacillus pumilus*. The 16S rRNA partial gene sequences of *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 has been registered in Nucleotide database of NCBI with GenBank accession numbers KJ530761 and KJ530762 respectively.

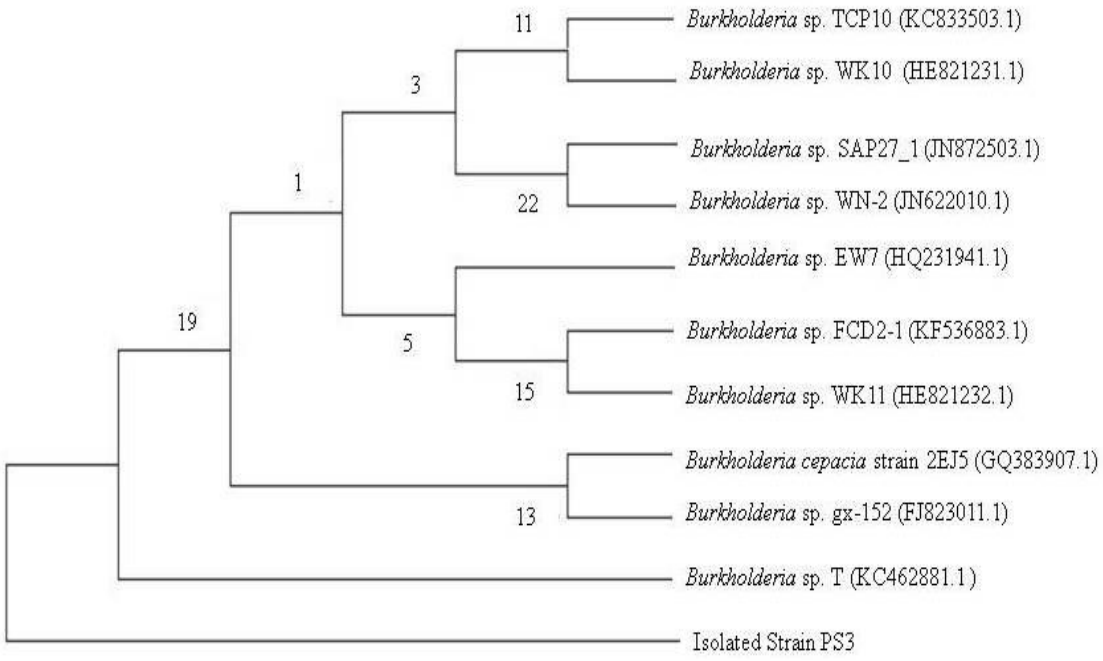


Fig.3.6. Phylogenetic tree for strain PS3

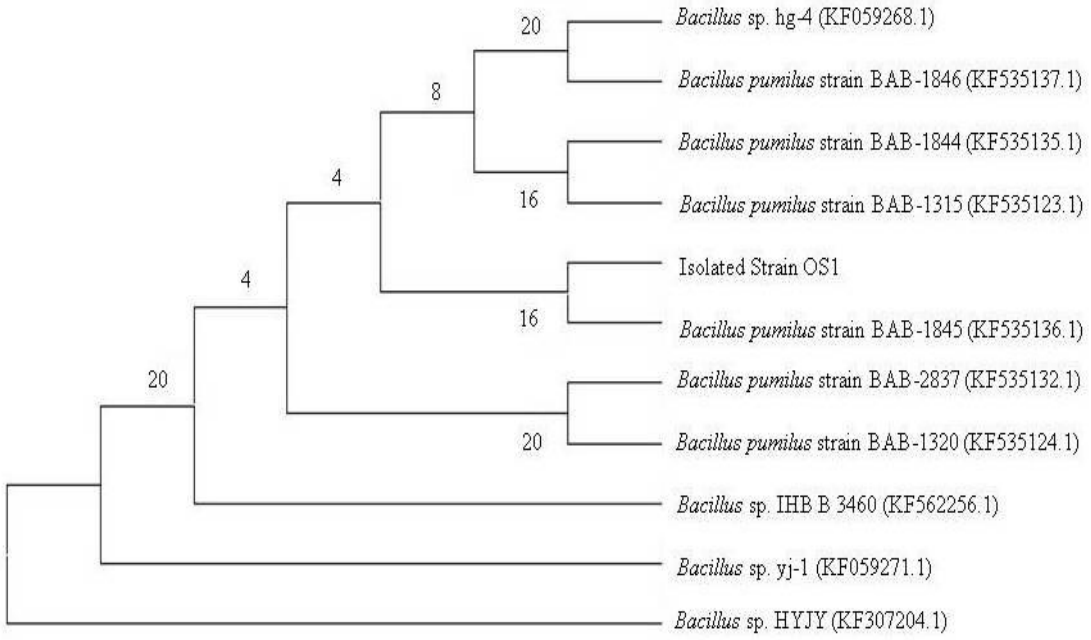


Fig.3.7. Phylogenetic tree for strain OS1

3.3 Optimization of medium components and physiological conditions for phenol degradation

Medium components and physiological conditions have effect on metabolism of microorganisms and hence optimization of these parameters is key step in many bioprocesses. Statistical methods play important role in quality and process improvement (Myers et al., 2009). Statistical experimental designs such as Plackett-Burman and

central composite design (CCD) can collectively optimize selected parameters to eliminate the limitations of a single-factor optimization process and hence they have been used for optimization of phenol biodegradation by isolated strains. Plackett-Burman design has been used for screening of significant factors from nine important variables for phenol degradation as the response and further, the significant factors have been optimized using central composite design. The statistical software Design Expert (Stat-Ease Inc., Minneapolis, USA) has been used for designing experiments and further analysis of response.

3.3.1 One Factor at a Time (OFAT) approach for determination of levels of variables:

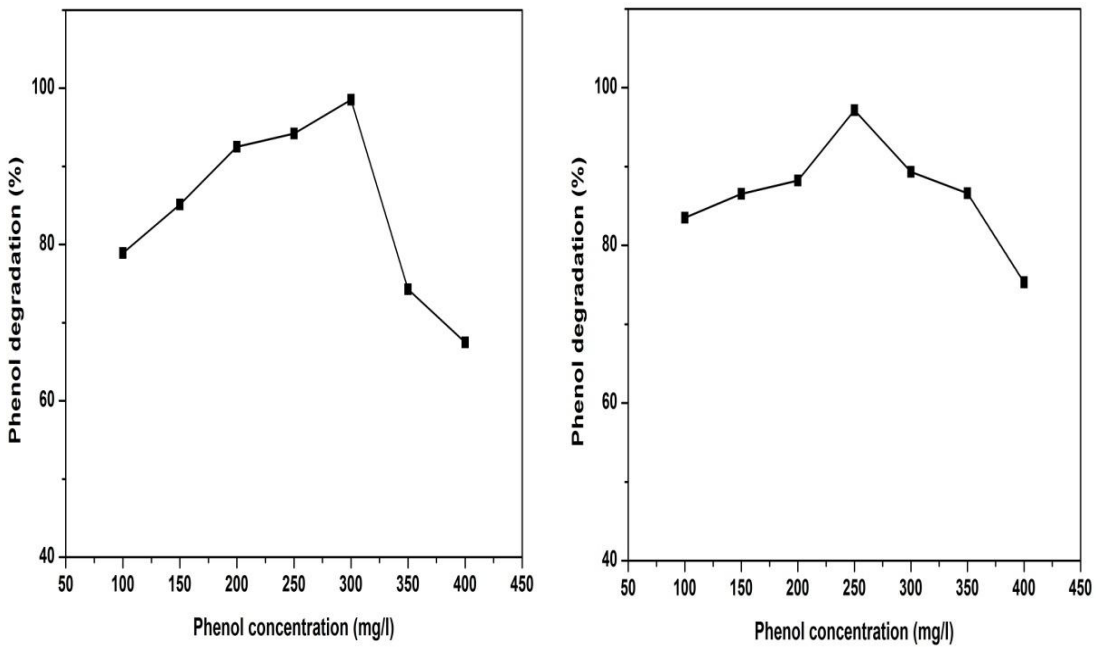
For both the isolated strains the media components except phenol have been used as composition mentioned in section 2.3.2. The flasks have been incubated for 30 hours and 36 hours for strain PS3 and OS1 respectively at 150 rpm.

3.3.1.1 Effect of initial phenol concentration:

To study effect of initial phenol concentration experiments have been performed at 100-400 mg/l phenol concentrations, pH 7, temperature 30°C and inoculum size 5% (v/v) for both the isolated strains independently. As shown in Fig.3.8 (A) and (B), 300 and 250 mg/l phenol concentration has been found optimum for strain PS3 and strain OS1 respectively. At phenol concentration higher than optimum, the percentage of phenol degradation decreased and this might be due to toxicity of phenol (Keweloh et al., 1990).

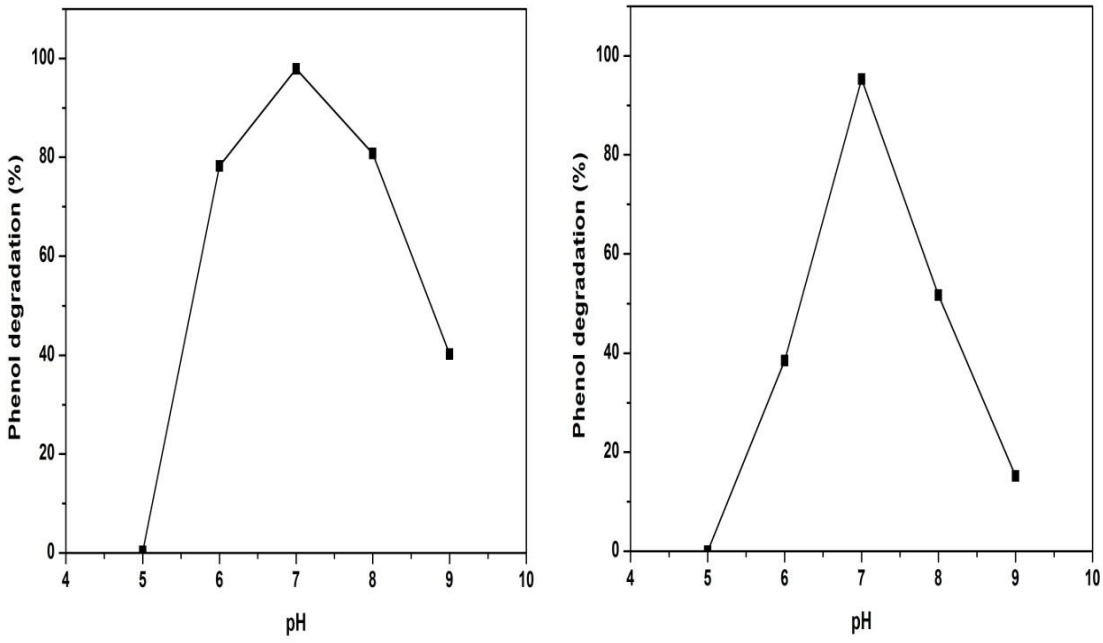
3.3.1.2 Effect of pH:

The experiments have been performed by varying pH as 5, 6, 7, 8, and 9 while keeping phenol concentration 300 and 250 mg/l for strains PS3 and OS1 respectively, temperature 30°C and inoculum size 5%, (v/v). For both the isolated strains maximum phenol degradation has been obtained at pH 7 (Fig.3.9 (A) and (B)). This indicates that the isolated strains are neutrophilic in nature. Isolated strains are found to be less effective in highly acidic or alkaline conditions and this might be due to enzyme activity completely reduced at extremely high or low pH values and at the optimum pH, the enzymes are most active and the microbes are stable (Banerjee and Ghoshal, 2010a). Previously, Lakshmi and Sridevi (2009) also reported maximum phenol degradation at pH 7.



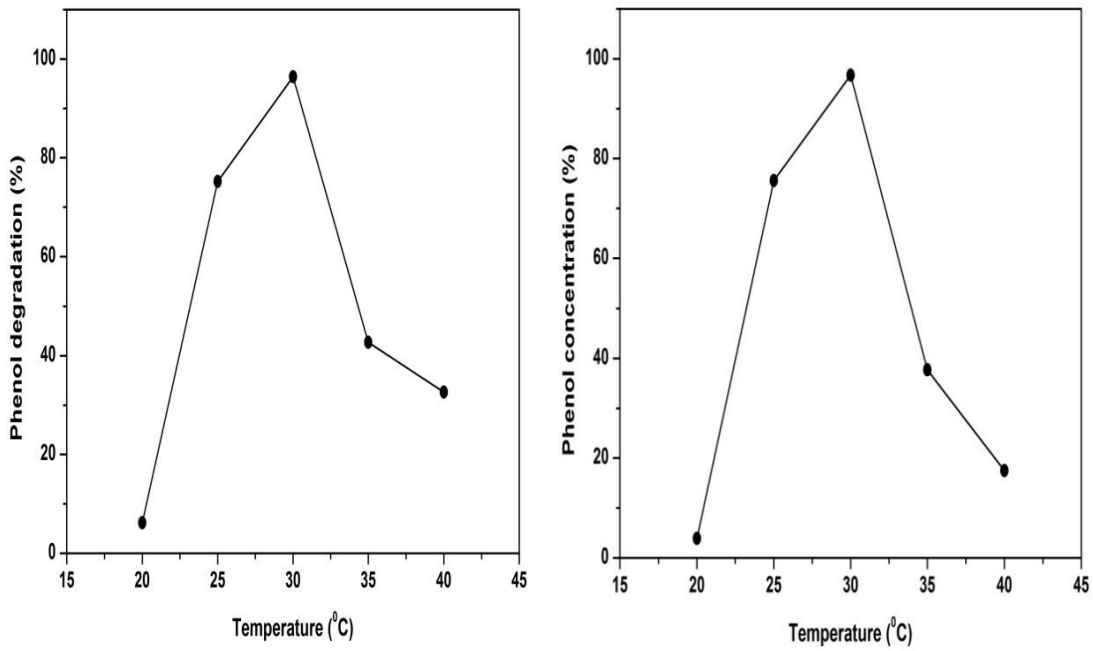
(A) (B)

Fig.3.8. Effect of initial concentration of phenol on phenol degradation by (A) *Burkholderia* sp. PS3 and (B) *Bacillus pumilus* OS1



(A) (B)

Fig.3.9. Effect pH on phenol degradation by (A) *Burkholderia* sp. PS3 and (B) *Bacillus pumilus* OS1



(A) (B)

Fig.3.10. Effect temperature on phenol degradation by (A) *Burkholderia* sp. PS3 and (B) *Bacillus pumilus* OS1

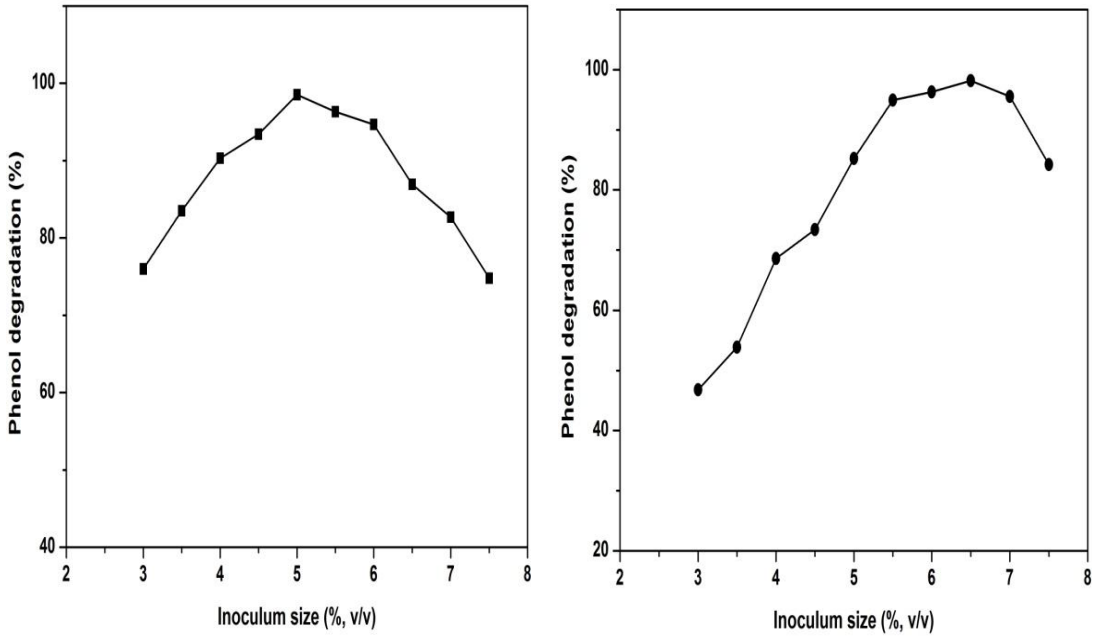
3.3.1.3 Effect of temperature:

In order to study effect of different temperature on degradation potential of the microbe, temperature has been varied within a range of 20-40°C with an interval of 5°C while keeping phenol concentration 300 and 250 mg/l for strains PS3 and OS1 respectively and at pH 7 with inoculum size 5%, (v/v). For both the isolated strains, at high and low temperature values phenol degradation decreases and at temperature 30°C maximum percentage of phenol degradation has been obtained (Fig.3.10 (A) and (B)) and this might be due to microbial enzyme activity is effective at optimum temperature (Peterson et al., 2007). This indicates that isolated strains are mesophilic in nature. Bayoumi and Abul-Hamd (2010) also reported 30°C temperature optimum for phenol biodegradation.

3.3.1.4 Effect of inoculum size:

Inoculum size plays vital role in phenol degradation behavior exhibited by the organism. Inoculum size for the study has been varied between 3-7.5% (v/v) while 300 and 250 mg/l phenol concentrations have been used for strains PS3 and OS1 respectively. Temperature 30°C and pH 7 has been maintained throughout the experiment. For strain PS3, maximum phenol degradation achieved at inoculum size 5% (v/v) (Fig.3.11 (A)) and for strain OS1, maximum phenol degradation obtained at inoculum size 6.5% (v/v)

(Fig.3.11 (B)). From the previous reports (Bandyopadhyay et al., 1998; Arutchelvan et al., 2006) it can be infered that the inoculum size between the range of 5-7% (v/v) is considered to be suitable for maximum phenol degradation



(A) (B)

Fig.3.11. Effect inoculum size on phenol degradation by (A) *Burkholderia* sp. PS3 and (B) *Bacillus pumilus* OS1

3.3.2 Screening of significant factors using Plackett-Burman design:

Plackett-Burman design provides a fast and effective way to identify the important factors among a large number of variables, thereby, saving time and maintaining convincing information on each parameter (Abdel-Fattah et al., 2005). A total of eleven variables (including two dummy variables) have been analyzed with regard to their effect on phenol degradation by using Plackett-Burman design. Isolated *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 have been individually inoculated in mineral salt media containing medium components and physiological conditions as per mentioned in Table 2.6 and 2.7 respectively. Percentage of phenol degradation has been estimated as a response for each experiment.

3.3.2.1 Screening of significant factors for isolated Burkholderia sp. PS3:

The percentage of phenol degradation by isolated *Burkholderia* sp. PS3 has been estimated for 16 experimental runs (as mentioned in Table 3.6). Analysis of the regression coefficients and the *p*-values of nine variables are shown in Table 3.7. Out of the nine important variables, temperature, phenol, inoculum size, KH_2PO_4 and $MgSO_4$

has positive effect while pH, K_2HPO_4, $(NH_4)_2SO_4$ and NaCl has negative effect as shown in Table 3.8. On the basis of *p*-value, it has been found that among nine variables the pH, temperature, phenol and inoculum size have significant effect on phenol degradation by *Burkholderia* sp. PS3 (Table 3.8). The model equation describing the correlation between nine variables and phenol degradation by *Burkholderia* sp. PS3 is as follows:

$$Y = 37.92 - 8.51X_1 + 4.48X_2 + 3.37X_3 + 4.72X_4 + 0.55X_5 - 0.78X_6 - 0.46X_7 - 0.06X_8 + 0.28X_9 \quad (3.1)$$

where, Y = response i.e. percentage of phenol degradation. The R^2 (Coefficient of determination) value closure to one represents the good statistical model. In presented model, R^2 has been 0.9658 which indicated upto 96.58% variability in phenol degradation could be calculated. The Predicted R^2 of 0.8806 has been in reasonable agreement with the Adjusted R^2 of 0.9043.

3.3.2.2 Screening of significant factors for isolated Bacillus pumilus OS1:

The percentage of phenol degradation by isolated *Bacillus pumilus* OS1 has been estimated for 16 experimental runs (Table 3.7). Analysis of the regression coefficients and the *p*-values of nine variables are shown in Table 3.9. Out of the nine variables, temperature, phenol concentration, inoculum size, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NaCl and $MgSO_4$ concentration has positive effect while pH has negative effect as shown in Table 3.9. On the basis of *p*-value, it has been found that among nine variables the pH, temperature, phenol, inoculum size and $(NH_4)_2SO_4$ have significant effect on phenol degradation by *Bacillus pumilus* OS1 (Table 3.9). The model equation describing the correlation between nine variables and phenol degradation by *Bacillus pumilus* OS1 is as follows:

$$Y = 38.59 - 8.39X_1 + 3.51X_2 + 4.79X_3 + 4.11X_4 + 1.71X_5 + 0.76X_6 + 5.11X_7 + 0.93X_8 + 1.13X_9 \quad (3.2)$$

where, Y = response i.e. percentage of phenol degradation. In presented model, R^2 has been 0.9752 which indicated upto 97.52% variability in phenol degradation could be calculated. The Predicted R^2 of 0.9129 has been in reasonable agreement with the Adjusted R^2 of 0.9306.

Table 3.6: Plackett-Burman design matrix for nine variables and experimentally determined percentage degradation of phenol by *Burkholderia* sp. PS3

pH	**Temperature** (^{0}C)	**Phenol** (mg/l)	**Inoculum size** (%, v/v)	**KH_2PO_4** (mg/l)	**K_2HPO_4** (mg/l)	**$(NH_4)_2SO_4$** (mg/l)	**NaCl** (mg/l)	**$MgSO_4$** (mg/l)	**Phenol degradation** (%)
8	35	200	8	300	500	300	50	50	37.73
6	35	400	2	300	500	500	50	50	46.17
8	25	400	8	100	500	500	150	50	31.46
6	35	200	8	300	300	500	150	150	53.91
6	25	400	2	300	500	300	150	150	43.54
6	25	200	8	100	500	500	50	150	41.27
8	25	200	2	300	300	500	150	50	16.80
8	35	200	2	100	500	300	150	150	22.66
8	35	400	2	100	300	500	50	150	35.12
6	35	400	8	100	300	300	150	50	58.78
8	25	400	8	300	300	300	50	150	32.65
6	25	200	2	100	300	300	50	50	34.89
7	30	300	5	200	400	400	100	100	93.60
7	30	300	5	200	400	400	100	100	96.80
7	30	300	5	200	400	400	100	100	98.40
7	30	300	5	200	400	400	100	100	98.32

Table 3.7: Plackett-Burman design matrix for nine variables and experimentally determined percentage degradation of phenol by *Bacillus pumilus* OS1

pH	**Temperature** (^{0}C)	**Phenol** (mg/l)	**Inoculum size** (%, v/v)	**KH_2PO_4** (mg/l)	**K_2HPO_4** (mg/l)	**$(NH_4)_2SO_4$** (mg/l)	**NaCl** (mg/l)	**$MgSO_4$** (mg/l)	**Phenol degradation** (%)
8	35	150	9.5	300	500	300	50	50	29.54
6	35	350	3.5	300	500	500	50	50	55.46
8	25	350	9.5	100	500	500	150	50	41.30
6	35	150	9.5	300	300	500	150	150	59.69
6	25	350	3.5	300	500	300	150	150	44.78
6	25	150	9.5	100	500	500	50	150	45.39
8	25	150	3.5	300	300	500	150	50	21.90
8	35	150	3.5	100	500	300	150	150	19.58
8	35	350	3.5	100	300	500	50	150	38.45
6	35	350	9.5	100	300	300	150	50	49.85
8	25	350	9.5	300	300	300	50	150	30.40
6	25	150	3.5	100	300	300	50	50	26.70
7	30	250	6.5	200	400	400	100	100	97.40
7	30	250	6.5	200	400	400	100	100	95.79
7	30	250	6.5	200	400	400	100	100	98.14
7	30	250	6.5	200	400	400	100	100	92.40

Table 3.8: Effects of the variables and statistical analysis of the Plackett-Burman design for *Burkholderia* sp. PS3

	Effect	**Coefficient**	**F value**	***p*-value**
Intercept		37.92	15.69	0.0037[a]
X_1-pH	-17.02	-8.51	80.35	0.0003[a]
X_2-Temperature	8.96	4.48	22.26	0.0053[a]
X_3-Phenol	6.74	3.37	12.61	0.0164[a]
X_4- Inoculum size	9.44	4.72	24.69	0.0042[a]
X_5-KH_2PO_4	1.10	0.55	0.34	0.5865
X_6-K_2HPO_4	-1.55	-0.78	0.67	0.4506
X_7-$(NH_4)_2SO_4$	-0.92	-0.46	0.23	0.6485
X_8-NaCl	-0.11	-0.06	0.0036	0.9547
X_9-MgSO4	0.55	0.28	0.08	0.7825

R^2 = 0.9658; Adj-R^2 = 0.9043; Pred-R^2 = 0.8806

[a] *p*-value less than 0.05 indicate model terms are significant

Table 3.9: Effects of the variables and statistical analysis of the Plackett-Burman design for *Bacillus pumilus* OS1

	Effect	**Coefficient**	**F value**	***p*-value**
Intercept		38.59	21.87	0.0017[a]
X_1-pH	-16.78	-8.39	89.84	0.0002[a]
X_2-Temperature	7.02	3.51	15.70	0.0107[a]
X_3-Phenol	9.57	4.79	29.23	0.0029[a]
X_4- Inoculum size	8.22	4.11	21.53	0.0056[a]
X_5-KH_2PO_4	3.42	1.71	3.72	0.1116
X_6-K_2HPO_4	1.51	0.76	0.73	0.4327
X_7-$(NH_4)_2SO_4$	10.22	5.11	33.33	0.0022[a]
X_8-NaCl	1.86	0.93	1.10	0.3416
X_9-MgSO4	2.26	1.13	1.62	0.2585

R^2 = 0.9752; Adj-R^2 = 0.9306; Pred-R^2 = 0.9129

[a] *p*-value less than 0.05 indicate model terms are significant

3.3.3 Optimization of screened factors by central composite design:

Central composite design has been used to study the interactions between the significant factors. The effect of each factor has been studied by three dimensional surface plots. These plots have been obtained using Design Expert software. The point prediction feature has been further has been used to determine optimum levels.

3.3.3.1 *Optimization of screened factors by central composite design for isolated Burkholderia* sp. PS3:

The design matrix of tested variables and the experimental results are represented in Table 3.10. The adequacy of model has been checked using Analysis of Variance (ANOVA) as shown in Table 3.11.

Table 3.10: Experimental design and results of CCD for actual factors for *Burkholderia* sp. PS3

Run	pH	Temperature (°C)	Phenol (mg/l)	Inoculum size (%, v/v)	Phenol degradation (%)	
					Observed	Predicted
1	6.5	28	250	3.5	77.23	81.01
2	7.5	28	250	3.5	71.50	70.98
3	6.5	32	250	3.5	51.66	50.76
4	7.5	32	250	3.5	34.74	38.04
5	6.5	28	350	3.5	83.38	85.52
6	7.5	28	350	3.5	84.12	81.18
7	6.5	32	350	3.5	42.54	46.01
8	7.5	32	350	3.5	36.94	38.99
9	6.5	28	250	6.5	67.98	64.70
10	7.5	28	250	6.5	81.73	80.71
11	6.5	32	250	6.5	58.73	64.12
12	7.5	32	250	6.5	80.84	77.46
13	6.5	28	350	6.5	56.81	55.96
14	7.5	28	350	6.5	78.00	77.67
15	6.5	32	350	6.5	46.85	46.13
16	7.5	32	350	6.5	66.49	65.16
17	6	30	300	5	78.81	74.90
18	8	30	300	5	81.20	83.89
19	7	26	300	5	77.42	79.54
20	7	34	300	5	40.10	36.77
21	7	30	200	5	88.22	87.14
22	7	30	400	5	79.48	79.35
23	7	30	300	2	34.96	30.38
24	7	30	300	8	36.87	40.24
25	7	30	300	5	94.94	96.59
26	7	30	300	5	98.48	96.59
27	7	30	300	5	98.10	96.59
28	7	30	300	5	98.54	96.59
29	7	30	300	5	98.35	96.59
30	7	30	300	5	91.12	96.59

Table 3.11: ANOVA for response surface quadratic model for *Burkholderia* sp. PS3

	Sum of Squares	df	Mean Square	F Value	*p*-value Prob > F
Model	12981.48	14	927.25	60.95	< 0.0001[a]
X_1-pH	121.32	1	121.32	7.97	0.0128[a]
X_2-Temperature	2743.48	1	2743.48	180.32	< 0.0001[a]
X_3-Phenol	91.10	1	91.10	5.99	0.0272[a]
X_4- Inoculum size	145.73	1	145.73	9.58	0.0074[a]
X_1X_2	7.18	1	7.18	0.47	0.5025
X_1X_3	32.38	1	32.38	2.13	0.1653

X_1X_4	678.60	1	678.60	44.60	< 0.0001[a]
X_2X_3	85.66	1	85.66	5.63	0.0315[a]
X_2X_4	881.20	1	881.20	57.92	< 0.0001[a]
X_3X_4	175.43	1	175.43	11.53	0.004[a]
X_1^2	506.61	1	506.61	33.30	< 0.0001[a]
X_2^2	2532.54	1	2532.54	166.46	< 0.0001[a]
X_3^2	305.33	1	305.33	20.07	0.0004[a]
X_4^2	6437.73	1	6437.73	423.14	< 0.0001[a]
Residual	228.21	15	15.21		
Lack of Fit	182.82	10	18.28	2.01	0.2276
Pure Error	45.40	5	9.08		
Cor Total	13209.69	29			

R^2 = 0.9827; Adj-R^2 = 0.9666; Pred-R^2 = 0.9153; Coefficient of variation = 5.53%

[a] *p*-value less than 0.05 indicate model terms are significant

The regression equation coefficients have been calculated and results of central composite design have been fitted with second order polynomial model equation. The regression equation of relationship to the phenol degradation by *Burkholderia* sp. PS3 with all terms regardless of their significance is shown as follows:

$$Y = 96.59 + 2.25X_1 - 10.69X_2 - 1.95X_3 + 2.46X_4 - 0.67X_1X_2 + 1.42X_1X_3 + 6.51X_1X_4 - 2.31X_2X_3 + 7.42X_2X_4 - 3.31X_3X_4 - 4.30X_1^2 - 9.61X_2^2 - 3.34X_3^2 - 15.32X_4^2 \quad (3.3)$$

The above model can be used to predict the percentage degradation of phenol within limits of experimental factors. The R^2 (Coefficient of determination) value for regression model has been 0.9827 indicating experimental results have been best fitted by quadratic model. Fig.3.12 shows the experimental response values agree well with predicted response values. The Predicted R^2 of 0.9153 has been in reasonable agreement with the Adjusted R^2 of 0.9666.

A *p*- value less than 0.0001 indicate that model has been statistically significant. The Lack of Fit F-value of 2.01 implies the Lack of Fit has been not significant relative to the pure error. There has been a 22.76% chance that a Lack of Fit F-value this large could occur due to noise. Adequate Precision measures the signal to noise ratio and it has been found as 24.005. Coefficient of variation is the standard deviation and it has been found as 5.53% and its lower value indicated that performed experiments have been highly reliable.

The effect of each factor has been studied by three dimensional surface plots. Each plot describes the effect of two parameters on the response (percentage of phenol degradation), keeping other factors at their zero levels. The effects of the pH (X_1) and

Temperature (X_2) on the response (Y) at fixed phenol concentration (X_3) of 300 mg/l and inoculum size (X_4) of 5% (v/v) are shown in Fig. 3.13(A).

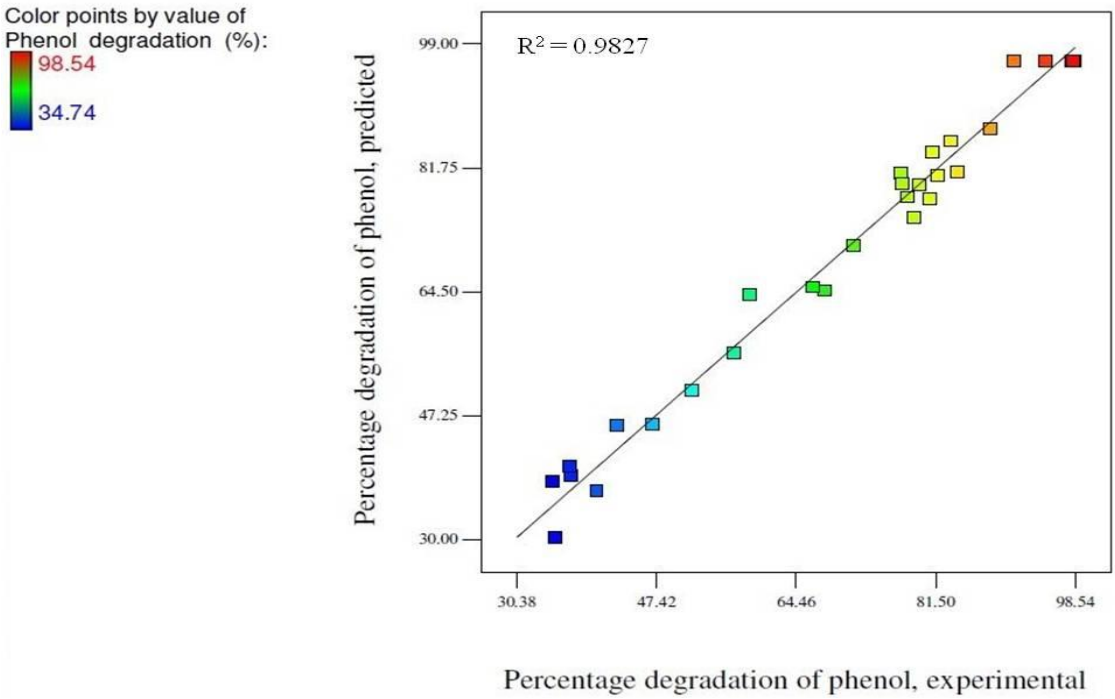
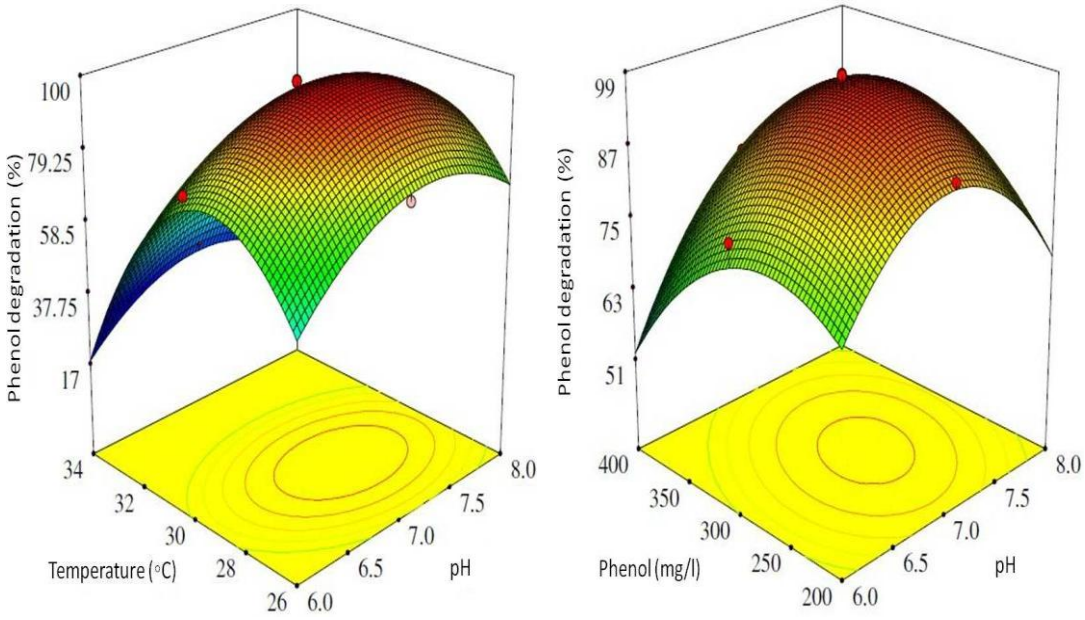


Fig.3.12. Predicted vs. Experimental percentage of phenol degradation by *Burkholderi*a sp. PS3

It has been found that the interaction between pH and temperature has been negligible as indicated by the shape of this three dimensional surface plot and *p*- value (> 0.05) (Table 3.11). The effects of pH (X_1) and phenol concentration (X_3) on the response (Y) while keeping temperature (X_2), and inoculum size (X_4) fixed are shown in Fig. 3.13(B). This three dimensional surface plot suggests that the interaction between pH and phenol has been negligible as indicated by nature of three dimensional surface plot high *p*- vlaue (> 0.05) (Table 3.11).

The effects of the pH (X_1) and inoculum size (X_4) on the response (Y) at fixed temperature (X_2) and phenol concentration (X_3) are shown in Fig. 3.14(A). Response (Y) increases as pH increased from 6 to 7.19 and then decreases with increase in pH. This indicates that the pH near to neutral is suitable for phenol biodegradation. Ullhyan and Ghosh (2012) also reported that phenol biodegradation occurs best near neutral pH due to neutrophilic behaviour of bacteria. Response (Y) increases as inoculum size increases from 2 to 5.24% (v/v) and further decreases with increase in inoculum size. This suggests that the increase in inoculum size beyond optimum level could not increase phenol consumption. Similarly, Lakshmi and Sridevi (2009) reported that the 5% (v/v)

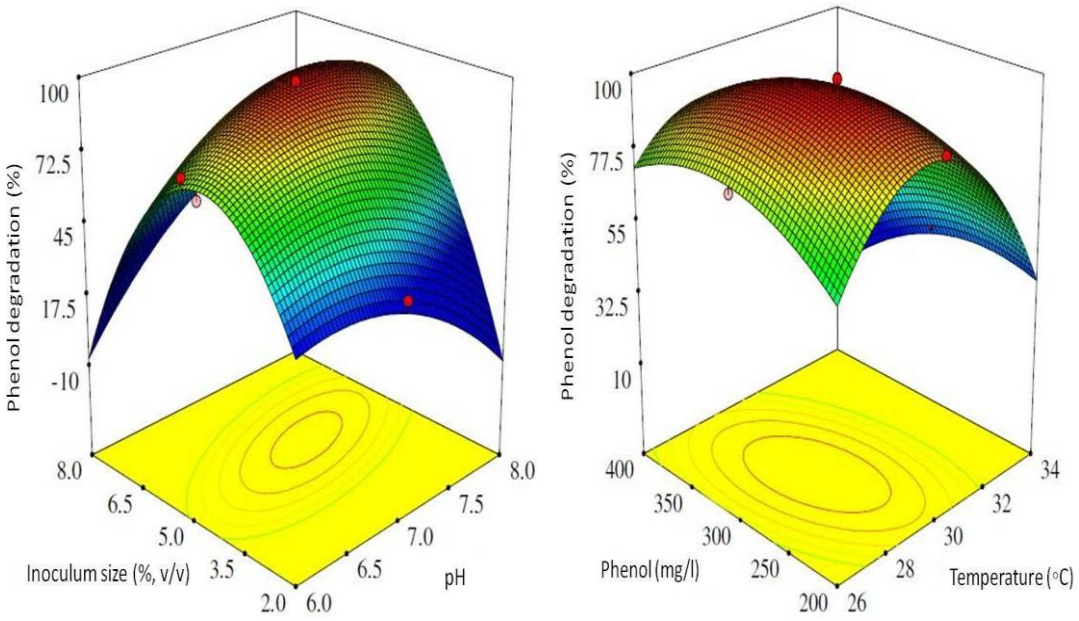
inoculum size has been optimum for phenol degradation by *Pseudomonas aeruginosa* while at higher inoculum sizes the phenol consumption rate decreased. At these conditions, maximum 97.07% of phenol degradation has been predicted. Thus, the effect of mutual interaction between pH and inoculum size has been significant on response as suggested by the nature of this three dimensional surface plot and low *p*- value (< 0.05) (Table 3.11)



(A) (B)

Fig.3.13. Three dimensional response surface plots of the effect of variable interactions on phenol degradation by *Burkholderia* sp. PS3 (A) pH and temperature; (B) pH and phenol.

Temperature (X_2) and phenol concentration (X_3) effects on the response (Y) while keeping pH (X_1) and inoculum size (X_4) at zero levels are shown in Fig. 3.14 (B). At these conditions, maximum 99.60% of phenol degradation has been predicted. The response (Y) increases as temperature increased from 26 to 28.9°C and then decreases with increase in temperature. The decrease in phenol degradation rate may be due to effective reactivity of multienzyme complex system within the cell (Bandyopadhyay et al., 1998). The response (Y) increases as concentration of phenol increases from 200 to 294.8 mg/l and then rapidly decreases with increase in phenol concentration. This prominent inhibition effect of phenol might be due to increase in initial phenol concentration (Agarry et al., 2008). The effect of mutual interaction between temperature and phenol has been significant on response as suggested by nature of three dimensional surface plot and low *p*-value (< 0.05) (Table 3.11).

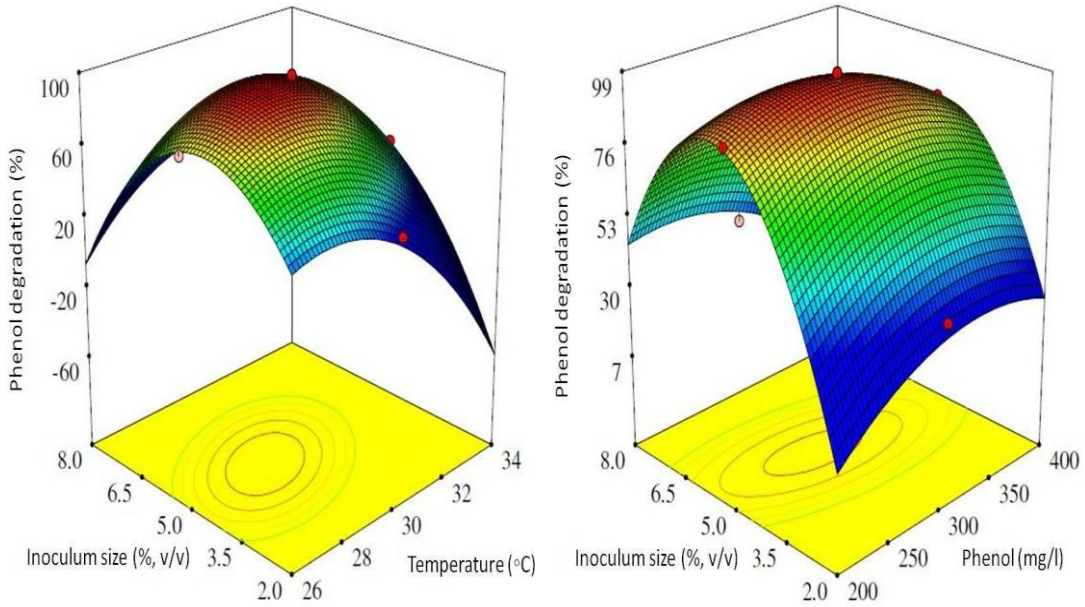


(A) (B)

Fig.3.14. Three dimensional response surface plots of the effect of variable interactions on phenol degradation by *Burkholderia* sp. PS3 (A) pH and inoculum size; (B) temperature and phenol.

The effects of temperature (X_2) and inoculum size (X_4) on the response (Y) at fixed pH (X_1) and phenol concentration (X_3) are shown in Fig. 3.15(A). The response increases as temperature and inoculum size increases to optimum conditions and then decreases with increase in these factors. The shape of this three dimensional plot and low *p*- value (< 0.05) indicated that the interaction between temperature and inoculum size has been significant on response. The effects of the phenol concentration (X_3) and inoculum size (X_4) on the response (Y) at fixed pH (X_1) and temperature (X_2) are shown in Fig. 3.15(B). The response increases as phenol concentration and inoculum size increases to optimum conditions and then decreases with increase in these factors. This three dimensional surface plot suggests that the effect of interaction between phenol and inoculum size has been significant on response as indicated by low *p*-value (< 0.05) (Table 3.11).

On the basis of response surface plots and applying point prediction feature, the maximum percentage of phenol degradation has been predicted at the following levels of factors: pH - 7.18, temperature - 28.9°C, phenol - 297.9 mg/l and inoculum size - 5.04% (v/v). Under these conditions the predicted phenol degradation has been 99.96%.



(A) (B)

Fig.3.15. Three dimensional response surface plots of the effect of variable interactions on phenol degradation by *Burkholderia* sp. PS3 (A) temperature and inoculum size; (B) phenol and inoculum size.

3.3.3.2 *Optimization of screened factors by central composite design for isolated Bacillus pumilus OS1:*

The design matrix of tested variables and the experimental results are represented in Table 3.12 and the adequacy of model has been checked using Analysis of Variance (ANOVA) as shown in Table 3.13.

Table 3.12: Experimental design and results of CCD for actual factors for *Bacillus pumilus* OS1

Run	**pH**	**Temperature** (°C)	**Phenol** (mg/l)	**Inoculum size** (%, v/v)	**$(NH_4)_2SO_4$** (mg/l)	**Phenol degradation** (%)	
						Observed	**Predicted**
1	6.5	28	200	5	350	72.80	76.62
2	7.5	28	200	5	350	72.17	72.14
3	6.5	32	200	5	350	68.90	61.92
4	7.5	32	200	5	350	40.04	49.76
5	6.5	28	300	5	350	74.95	76.81
6	7.5	28	300	5	350	72.61	71.99
7	6.5	32	300	5	350	51.14	55.24
8	7.5	32	300	5	350	47.90	42.74
9	6.5	28	200	8	350	59.60	53.16
10	7.5	28	200	8	350	75.34	74.94
11	6.5	32	200	8	350	46.49	50.93
12	7.5	32	200	8	350	68.14	65.02
13	6.5	28	300	8	350	48.11	47.54

14	7.5	28	300	8	350	62.61	68.98
15	6.5	32	300	8	350	36.48	38.43
16	7.5	32	300	8	350	54.62	52.19
17	6.5	28	200	5	450	66.87	70.63
18	7.5	28	200	5	450	69.67	67.57
19	6.5	32	200	5	450	57.20	54.15
20	7.5	32	200	5	450	45.17	43.41
21	6.5	28	300	5	450	67.30	68.09
22	7.5	28	300	5	450	65.82	64.70
23	6.5	32	300	5	450	44.50	44.75
24	7.5	32	300	5	450	25.90	33.67
25	6.5	28	200	8	450	54.10	53.98
26	7.5	28	200	8	450	75.03	77.19
27	6.5	32	200	8	450	46.54	49.97
28	7.5	32	200	8	450	68.96	65.49
29	6.5	28	300	8	450	46.91	45.64
30	7.5	28	300	8	450	68.79	68.50
31	6.5	32	300	8	450	38.53	34.75
32	7.5	32	300	8	450	48.97	49.94
33	6	30	250	6.5	400	39.80	39.79
34	8	30	250	6.5	400	52.65	50.49
35	7	26	250	6.5	400	74.32	72.51
36	7	34	250	6.5	400	39.60	39.25
37	7	30	150	6.5	400	90.50	91.66
38	7	30	350	6.5	400	79.61	76.29
39	7	30	250	3.5	400	78.41	73.87
40	7	30	250	9.5	400	64.31	66.68
41	7	30	250	6.5	300	79.52	77.35
42	7	30	250	6.5	500	69.09	69.10
43	7	30	250	6.5	400	98.56	97.14
44	7	30	250	6.5	400	96.20	97.14
45	7	30	250	6.5	400	98.28	97.14
46	7	30	250	6.5	400	98.30	97.14
47	7	30	250	6.5	400	98.24	97.14
48	7	30	250	6.5	400	98.18	97.14
49	7	30	250	6.5	400	89.10	97.14
50	7	30	250	6.5	400	98.10	97.14

The regression equation coefficients have been calculated and results of central composite design have been fitted with second order polynomial model equation. The regression equation coefficients have been calculated and results of central composite design have been fitted with second order polynomial model equation. The regression equation has been derived for phenol degradation by *Bacillus pumilus* OS1 as follows:

$$Y = 97.14 + 2.68X_1 - 8.32X_2 - 3.84X_3 - 1.80X_4 - 2.06X_5 - 1.92X_1X_2 - 0.085X_1X_3 + 6.57X_1X_4 + 0.36X_1X_5 - 1.72X_2X_3 + 3.12X_2X_4 - 0.44X_2X_5 - 1.45X_3X_4 - 0.68X_3X_5 + 1.70X_4X_5 - 13X_1^2 - 10.32X_2^2 - 3.29X_3^2 - 6.72X_4^2 - 5.98X_5^2 \quad (3.4)$$

Table 3.13: ANOVA for response surface quadratic model for *Bacillus pumilus* OS1

	Sum of Squares	**df**	**Mean Square**	**F Value**	***p*-value Prob > F**
Model	17777.23	20	888.86	43.69	< 0.0001[a]
X_1-pH	286.33	1	286.33	14.07	0.0008[a]
X_2-Temperature	2766.23	1	2766.23	135.98	< 0.0001[a]
X_3-Phenol	590.28	1	590.28	29.02	< 0.0001[a]
X_4-Inoculum size	129.31	1	129.31	6.36	0.0174[a]
X_5-$(NH_4)_2SO_4$	170.16	1	170.16	8.36	0.0072[a]
X_1X_2	118.12	1	118.12	5.81	0.0225[a]
X_1X_3	0.23	1	0.23	0.011	0.9158
X_1X_4	1379.18	1	1379.18	67.79	< 0.0001[a]
X_1X_5	4.06	1	4.06	0.20	0.6583
X_2X_3	94.26	1	94.26	4.63	0.0398[a]
X_2X_4	310.50	1	310.50	15.26	0.0005[a]
X_2X_5	6.34	1	6.34	0.31	0.5811
X_3X_4	67.51	1	67.51	3.32	0.0788
X_3X_5	14.80	1	14.80	0.73	0.4007
X_4X_5	92.89	1	92.89	4.57	0.0412[a]
X_1^2	5407.58	1	5407.58	265.81	< 0.0001[a]
X_2^2	3405.27	1	3405.27	167.39	< 0.0001[a]
X_3^2	346.79	1	346.79	17.05	0.0003[a]
X_4^2	1443.24	1	1443.24	70.94	< 0.0001[a]
X_5^2	1144.14	1	1144.14	56.24	< 0.0001[a]
Residual	589.96	29	20.34		
Lack of Fit	517.15	22	23.51	2.26	0.1355
Pure Error	72.82	7	10.40		
Cor Total	18367.19	49			

R^2 = 0.9679; Adj-R^2 = 0.9457; Pred-R^2 = 0.8946; Coefficient of variation = 6.87%

[a] *p*-value less than 0.05 indicate model terms are significant

The above model can be used to predict the percentage degradation of phenol within limits of experimental factors. R^2 value for regression model has been 0.9679 indicating experimental results have been best fitted by quadratic model. Fig.3.16 shows the experimental response values agree well with predicted response values obtained for phenol degradation by *Bacillus pumilus* OS1. The Predicted R^2 of 0.8946 has been in reasonable agreement with the R^2 adjusted of 0.9457.

The Lack of Fit F-value of 2.26 implies the Lack of Fit has been not significant relative to the pure error. There has been a 13.55% chance that a Lack of Fit F-value this large could occur due to noise. Adequate Precision measures the signal to noise ratio and it has

been found as 21.71 indicated an adequate signal. Coefficient of variation has been found as 6.87% and its lower value indicated that performed experiments have been highly dependable.

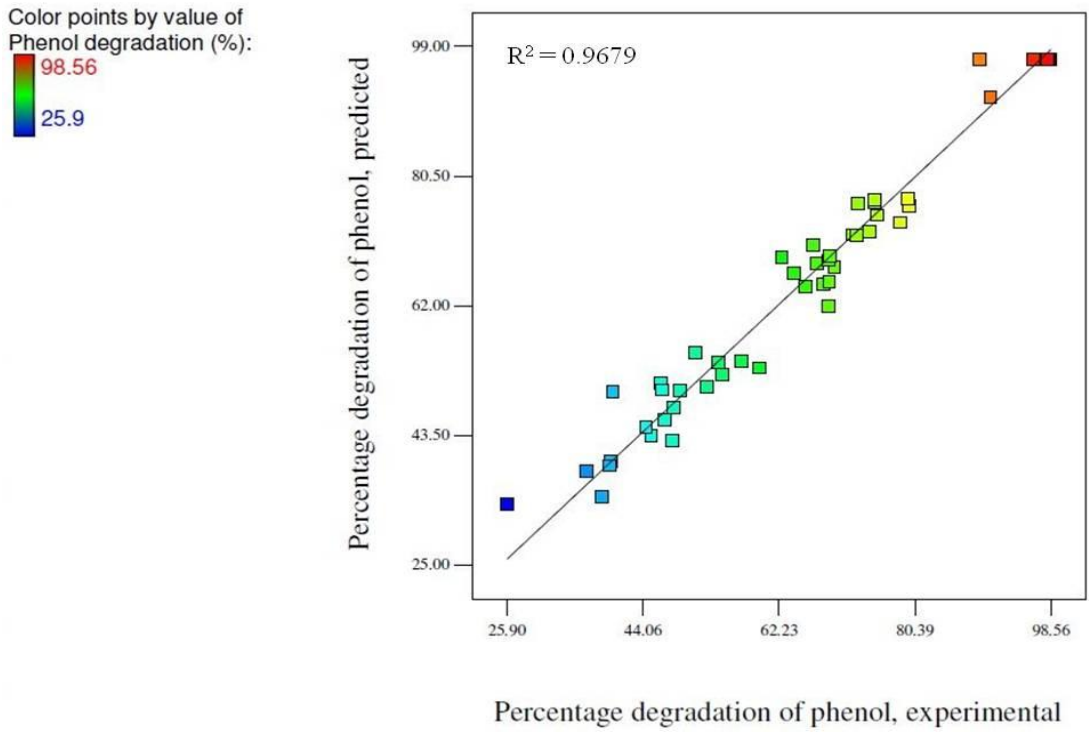


Fig.3.16. Predicted vs. Experimental percentage of phenol degradation by *Bacillus pumilus* OS1

The three dimensional surface plots have been constructed for study of effect of interation between parameters on the response. The effects of the pH (X_1) and temperature (X_2) on the response (Y) at fixed phenol concentration (X_3) of 250 mg/l, inoculum size (X_4) of 6.5% (v/v) and $(NH_4)_2SO_4$ (X_5) 400 mg/l are shown in Fig. 3.17(A). The response (Y) increases as pH increased from 6 to 7.07 and temperature increases from 26 to 29.2°C then response decreases with increase in pH and temperature. The decrease in response (Y) might be due to change in pH and temperature affects the solubility and reactivity of enzymatic compounds produced by microbes (Banerjee and Ghoshal, 2010a; Bandyopadhyay et al., 1998). At these conditions, maximum 99.04% of phenol degradation has been predicted. This three dimensional surface plot shows that the effect of interaction between pH and temperature has been significant on response as indicated by low *p*-value (<0.05) (Table 3.13).

The effects of pH (X_1) and phenol concentration (X_3) on the response (Y) while keeping temperature (X_2), inoculum size (X_4) and $(NH_4)_2SO_4$ (X_5) at their middle level are shown in Fig. 3.17(B). This three dimensional surface plot and *p*-value > 0.05 (Table 3.13)

suggested that the interaction between pH and phenol has been negligible. The effects of the pH (X_1) and inoculum size (X_4) on the response (Y) at fixed temperature (X_2), phenol concentration (X_3) and $(NH_4)_2SO_4$ (X_5) are shown in Fig. 3.17(C). Response (Y) increases as inoculum size increased from 3.5 to 6.36% (v/v) and further decreases with increase ininoculum size. This might be due to the increase in inoculum size reduces lag phase duration and beyond optimal value its effect become marginal (Arutchelvan et al, 2006). Response (Y) increases as pH increased from 6 to 7.04 and further decreases with increase in pH. At these conditions, the predicted maximum phenol degradation has been 97.33%. The nature of this three dimensional surface plot indicated that the mutual interaction between pH and inoculum size has been significant on response as suggested by low *p*- value (< 0.05) (Table 3.13). The effects of the pH (X_1) and $(NH_4)_2SO_4$ (X_5) on the response (Y) at fixed temperature (X_2), phenol concentration (X_3) and inoculum size (X_4) are shown in Fig. 3.17(D). This three dimensional surface plot and *p*- value (> 0.05) (Table 3.13) indicates that the effect of interaction between pH and $(NH_4)_2SO_4$ has been insignificant on response (Y).

Temperature (X_2) and phenol concentration (X_3) effects on the response (Y) while keeping pH (X_1), inoculum size (X_4) and $(NH_4)_2SO_4$ (X_5) at zero levels are shown in Fig. 3.18(A). The response (Y) increases as concentration of phenol has been increased from 150 to 225.6 mg/l and further rapidly decreases with increase in phenol concentration. The quick decrease of response might be due to inhibition effect phenol as a substrate (Scragg, 2006). The response (Y) increases as temperature has been increased from 28 to 29.3°C and further decreases with increase in temperature. At these conditions, the maximum 97.33% phenol degradation has been predicted. The mutual interaction between temperature and phenol have significant effect on response as indicated by this three dimensional plot and low *p*-value (<0.05) (Table 3.13). The effects of temperature (X_2) and inoculum size (X_4) on the response (Y) at fixed pH (X_1), phenol concentration (X_3) and $(NH_4)_2SO_4$ (X_5) are shown in Fig. 3.18(B). This three dimensional surface plot and low *p*-value (<0.05) (Table 3.13) suggested that the effect of mutual interaction between temperature and inoculum size has been siginficant on response.

Temperature (X_2) and $(NH_4)_2SO_4$ (X_5) effects on the response (Y) while keeping pH (X_1), phenol concentration (X_3) and inoculum size (X_4) at zero levels are shown in Fig. 3.18(C). This three dimensional surface plot and *p*-value > 0.05 (Table 3.13) indicated

that the effect of interaction between temperature and $(NH_4)_2SO_4$ has been insignificant on response.

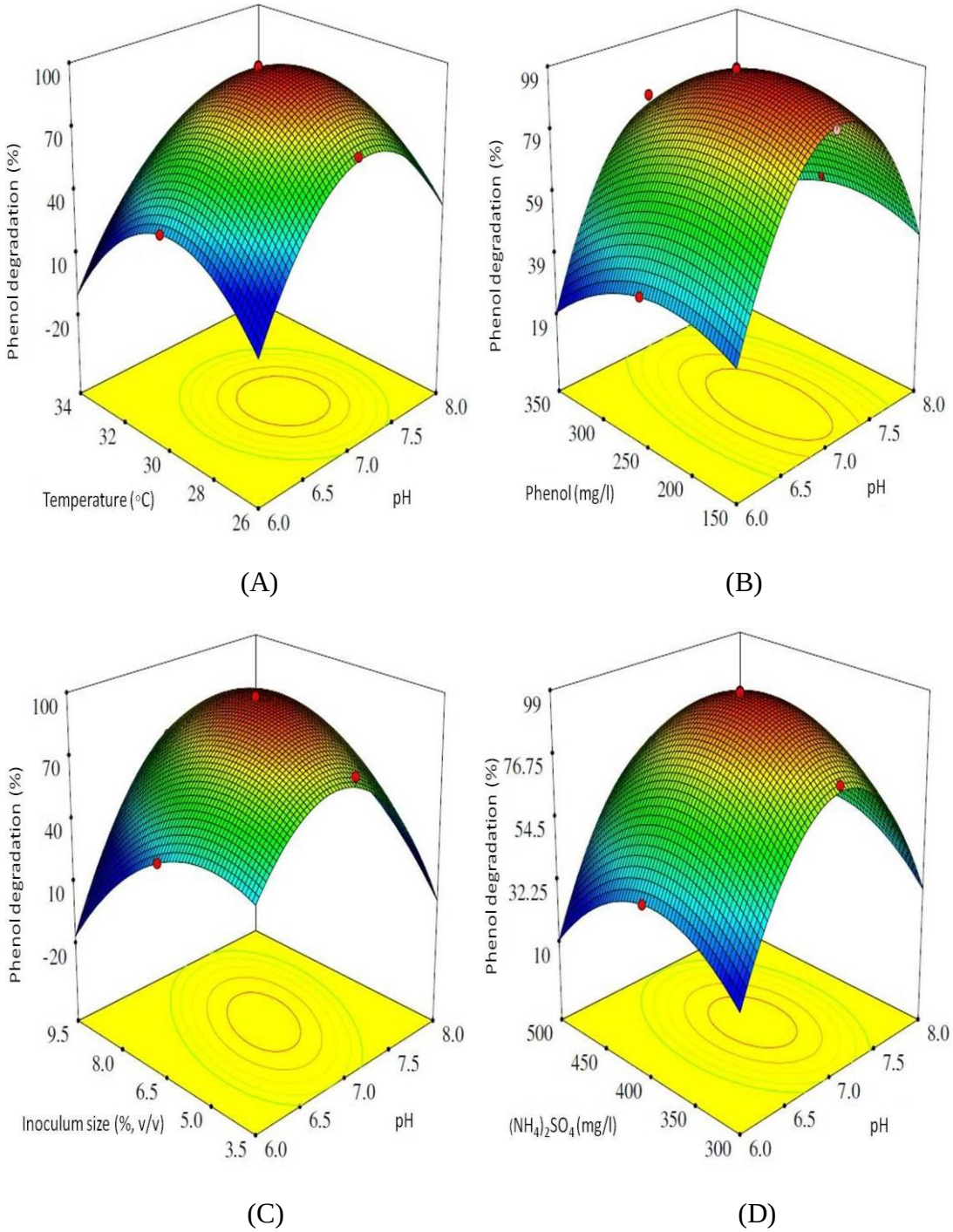


Fig.3.17. Three dimensional response surface plots of the effect of variable interactions on phenol degradation by *Bacillus pumilus* OS1 (A) pH and temperature; (B) pH and phenol; (C) pH and inoculum size; (D) pH and $(NH_4)_2SO_4$.

The effects of the phenol concentration (X_3) and inoculum size (X_4) on the response (Y) at fixed pH (X_1), temperature (X_2) and $(NH_4)_2SO_4$ (X_5) are shown in Fig. 3.18(D). The effect of interaction between phenol and inoculum size has been insiginificant on

response as indicated by nature of this three dimensional surface plot and *p*-value > 0.05 (Table 3.13).

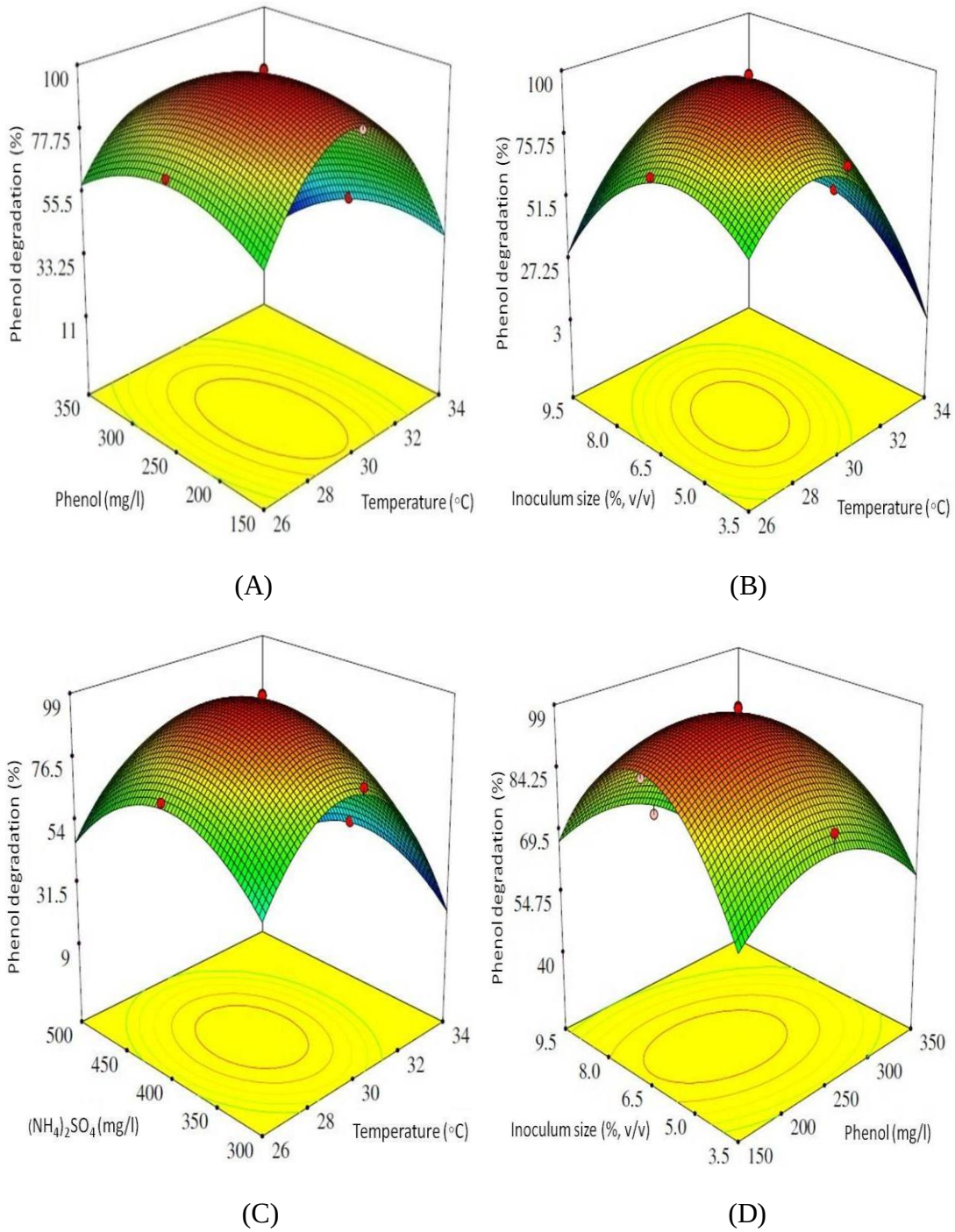
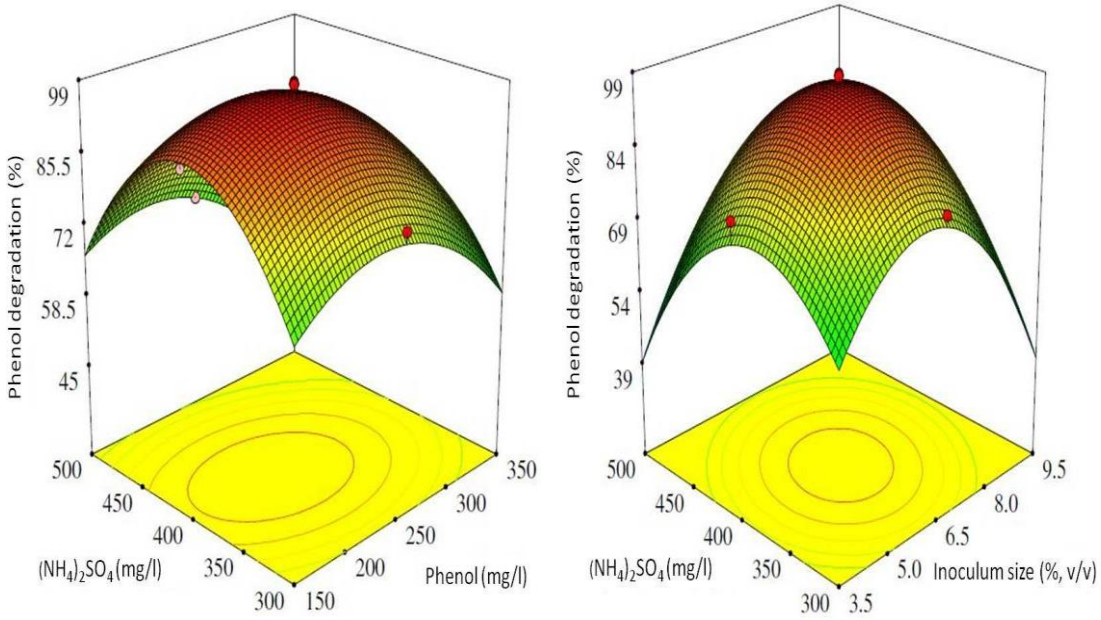


Fig.3.18. Three dimensional response surface plots of the effect of variable interactions on phenol degradation by *Bacillus pumilus* OS1 (A) temperature and phenol; (B) temperature and inoculum size; (C) temperature and $(NH_4)_2SO_4$; (D) phenol and inoculum size.

The effects of the phenol concentration (X_3) and $(NH_4)_2SO_4$ (X_5) on the response (Y) at fixed pH (X_1), temperature (X_2) and inoculum size (X_4) are shown in Fig. 3.19(A). From this three dimensional surface plot, it has been found that the effect of interaction

between phenol and $(NH_4)_2SO_4$ has been insignificant on response and these have been independent factors as indicated by *p*-value (>0.05) (Table 3.13). Inoculum size (X_4) and $(NH_4)_2SO_4$ (X_5) effects on the response (Y) while keeping pH (X_1), temperature (X_2) and phenol concentration (X_3) at zero levels are shown in Fig. 3.19(B). The response increases as the concentration of $(NH_4)_2SO_4$ increases from 300 to 390.3 mg/l and response further decreases with increase in $(NH_4)_2SO_4$ concentration. Previously, Annadurai et al, (2008) also reported that the addition of optimum concentration of $(NH_4)_2SO_4$ in media significantly decreases toxicity of phenol and in turn enhances cell.growth. The response increases as the inoculum size has been increases from 3 5 to 6.26 %, (v/v) and further decreases with increase in inoculum size. At these conditions, maximum predicted phenol degradation has been 97.48%. This three dimensional surface plot suggests that the effect of interaction between inoculum size and $(NH_4)_2SO_4$ has been significant on response as indicated by low *p*-value (<0.05) (Table 3.13).



(A) (B)

Fig.3.19. Three dimensional response surface plots of the effect of variable interactions on phenol degradation by *Bacillus pumilus* OS1 (A) phenol and $(NH_4)_2SO_4$; (B) inoculum size and $(NH_4)_2SO_4$.

The optimum levels of each variable for maximum percentage of phenol degradation have been determined on the basis of response surface plots and by applying point prediction feature and these have been as follows: pH - 7.07, temperature - 29.3°C, phenol - 227.4 mg/l, inoculum size - 6.3% (v/v) and $(NH_4)_2SO_4$ - 392.1 mg/l. Under these optimized conditions, predicted phenol degradation has been 99.99%.

3.4 Experimental validation of predicted model

Experimental validation of predicted model for both the isolated strains has been performed independently. As shown in Fig.3.20 and Fig.3.21, growth profile for both the isolated strains suggested that growth have been accelerated at optimum conditions and negligible lag phase duration has been observed and this might be due to absence of inhibition effect of phenol (Suhaila et al., 2013; Marrot et al., 2006).

3.4.1 Validation of predicted model for phenol degradation by *Burkholderia sp.* PS3:

Validation of obtained statistical model has been done by performing phenol degradation experiments at predicted levels of significant factors i.e. pH - 7.18, temperature - 28.9°C, phenol - 297.9 mg/l and inoculum size - 5.04% (v/v). At these optimum conditions, the predicted phenol degradation has been 99.96% and the average of experimental values has been 99.88% (Fig.3.20). Experimental value has been close to predicted value and that represented the validity of model.

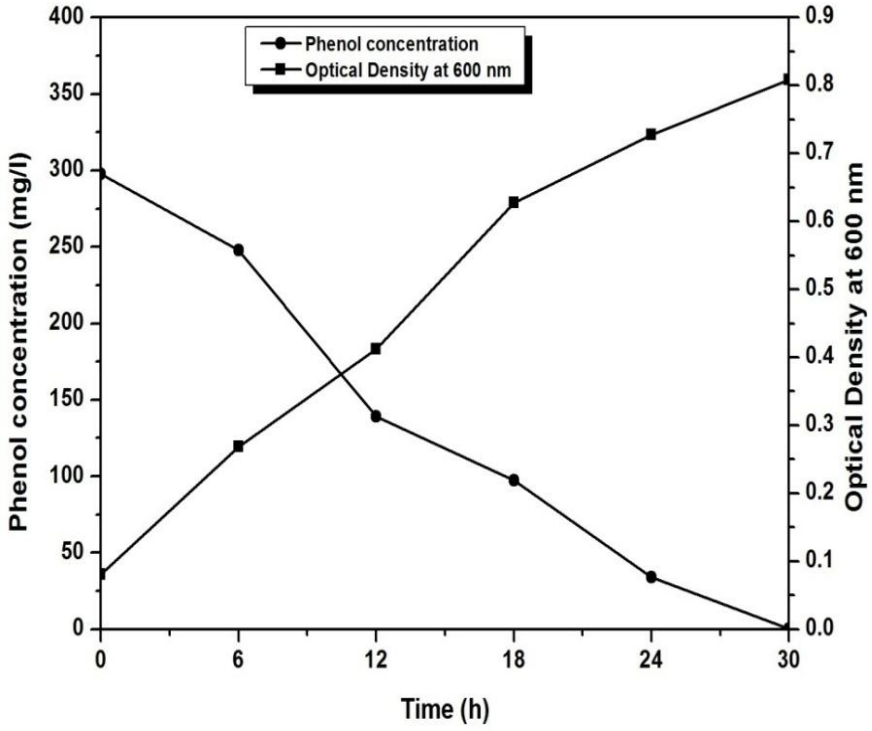


Fig.3.20. Phenol degradation and Growth profile for *Burkholderia sp*. PS3 at optimized conditions.

3.4.2 Validation of predicted model for phenol degradation by *Bacillus pumilus* OS1:

Validation of obtained statistical model has been done by performing phenol degradation experiments at predicted levels of significant factors i.e. pH - 7.07, temperature - 29.3°C,

phenol - 227.4 mg/l, inoculum size - 6.3% (v/v) and $(NH_4)_2SO_4$ - 392.1 mg/l. At these optimum conditions, the predicted response has been 99.99% and the average of experimental values has been 99.90% (Fig.3.21). Experimental value has been close to predicted value and that validated the model.

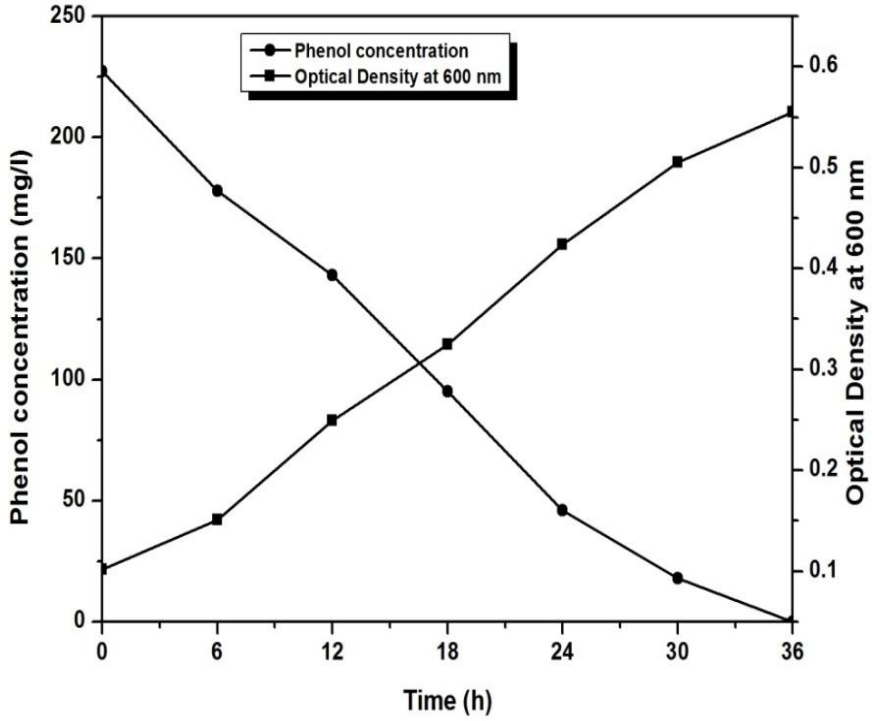


Fig.3.21. Phenol degradation and Growth profile for *Bacillus pumilus* OS1 at optimized conditions

3.5 Study of biodegradation of phenol

Growth and phenol degradation behavior of isolated *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 has been carried out in minimal salt media broth in batch mode. The strains have been individually inoculated into mineral salt media and incubated at optimized growth conditions and 150 rpm. The objective of this study has been to find out behavior of isolated strains to the increasing concentration of phenol.

3.5.1 Degradation study of phenol by *Burkholderia sp*. PS3:

Phenol degradation behavior of *Burkholderia* sp. PS3 has been studied at 500-1500 mg/l phenol concentration with an interval of 250 mg/l and 1500-1700 mg/l with an interval of 50 mg/l. The other parameters have been kept at their optimized level i.e. pH - 7.18, temperature - 28.9°C and inoculum size - 5.04% (v/v). Fig.3.22 and 3.23 show growth curves of *Burkholderia* sp. PS3 at various initial phenol concentrations.

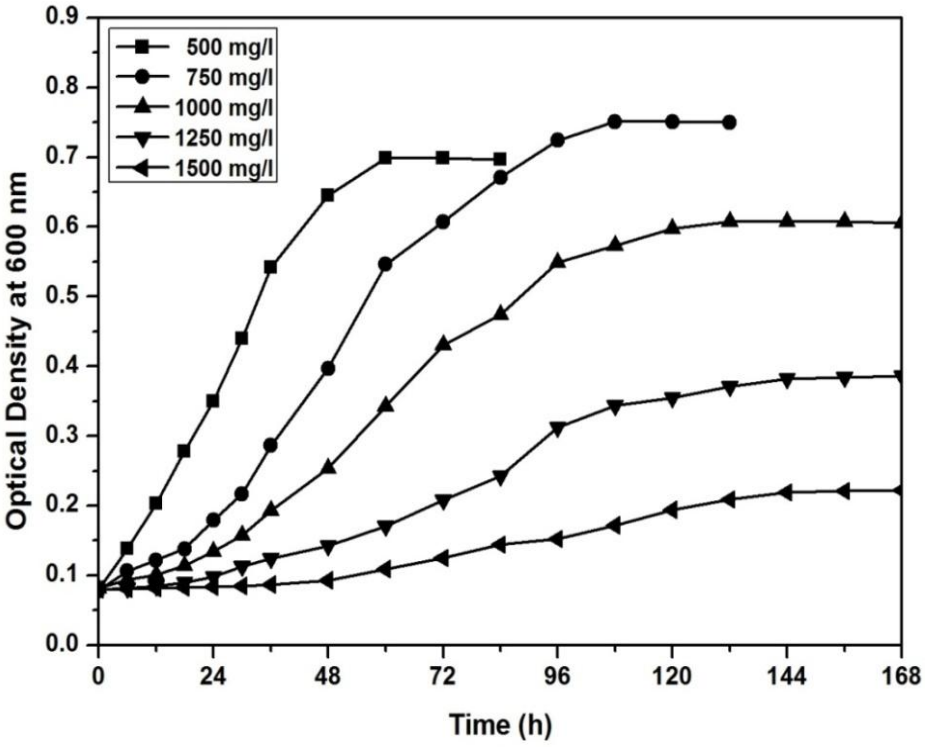


Fig.3.22. Growth profile for *Burkholderia sp*. PS3 at various initial phenol concentration

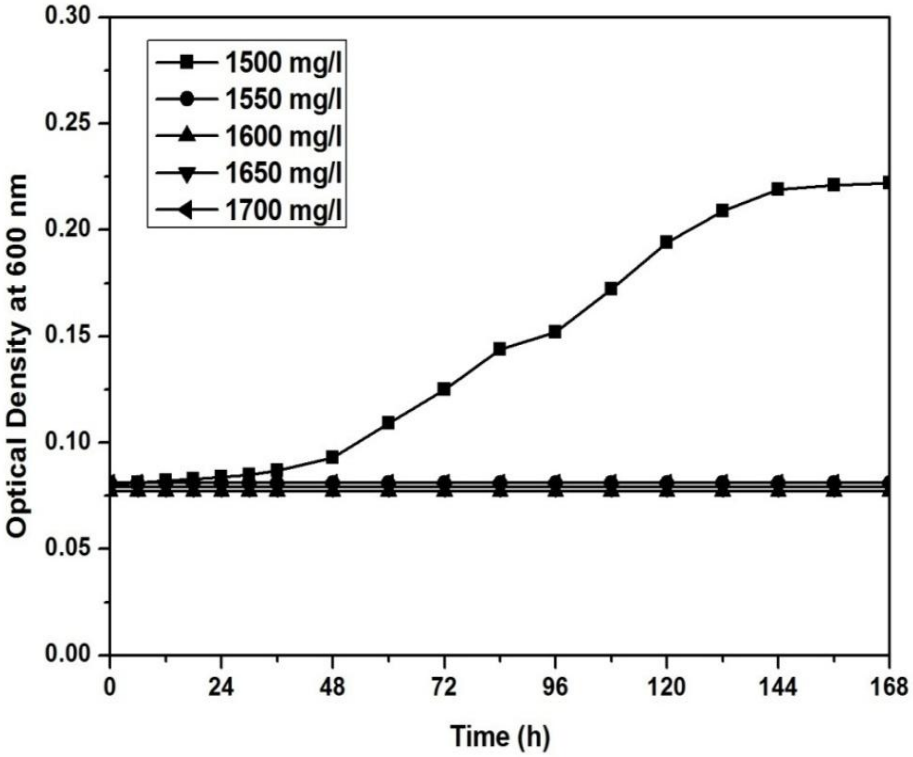


Fig.3.23. Growth profile for *Burkholderia sp*. PS3 at various initial higher phenol concentration

Fig.3.22 represents, at phenol concentrations 500 mg/l, there is no inhibitory effect of phenol as lag phase has been not observed. At phenol concentrations higher than 500 mg/l, lag phase has been observed and lag phase duration has been increased as initial

phenol concentration increased. As shown in Fig.3.23, from phenol concentration 1550 mg/l and above the microbes growth has not been observed and thus the microbes have been completely inhibited.

Fig. 3.24 represents phenol degradation profile for *Burkholderia* sp. PS3 at various initial phenol concentrations. The 98.86%, 98.62%, 83.3%, 51.2% and 17.3% degradation of 500, 750, 1000, 1250 and 1500 mg/l phenol concentrations has been achieved.

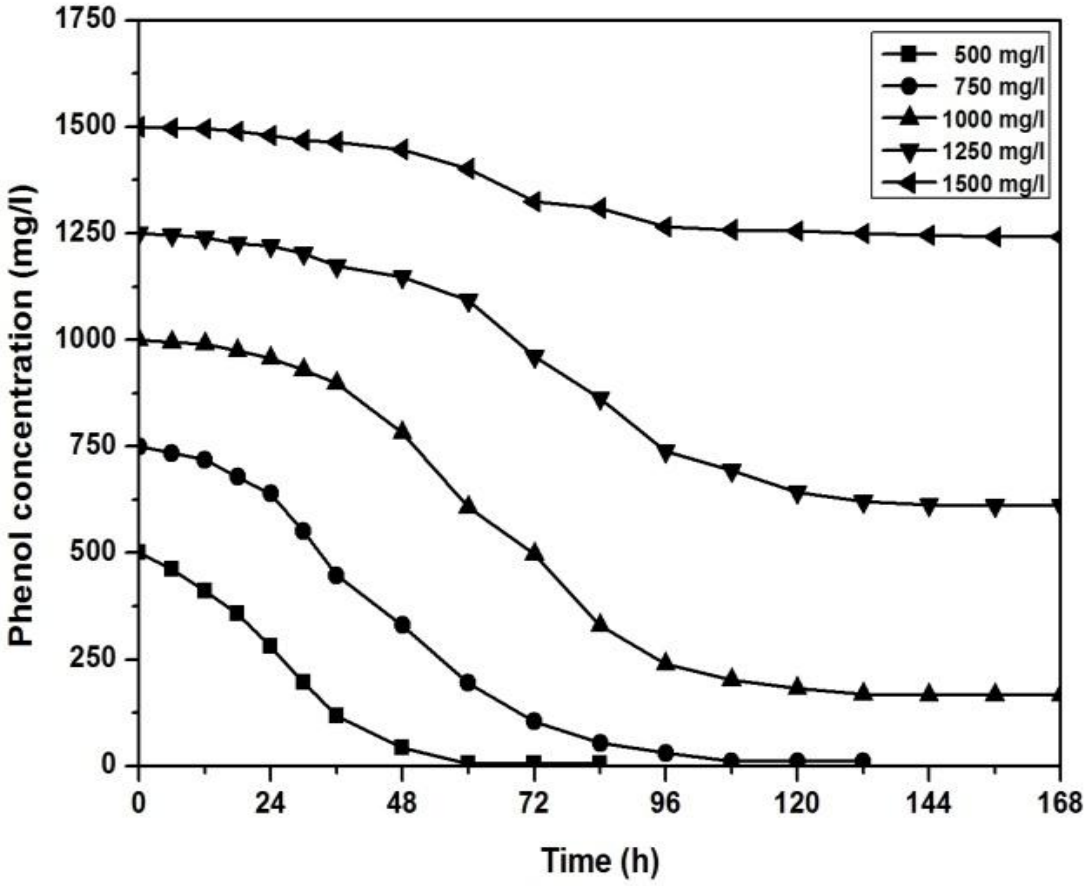


Fig.3.24. Phenol degradation profile for *Burkholderia sp*. PS3 at various initial phenol concentrations

Fig.3.25 shows Phenol degradation profile for *Burkholderia* sp. PS3 at various initial higher phenol concentrations. The figure infers that the microbe is able to degrade up to 1500 mg/l of phenol and gets completely inhibited above this concentration. Previously, very few work has been reported on phenol degradation by *Burkholderia* sp. Cobos-Vasconcelos et al. (2006) have reported cometabolic degradation of 500 mg/l phenol and various chlorophenols concentration by *Burkholderia tropicalis* in fed batch cultivation. But in present study, for isolated *Burkholderia* sp. PS3, the degradation experiments have been performed in batch mode with phenol as sole source of carbon. Thus, isolated *Burkholderia* sp. PS3 has been found to be an efficient phenol degrading microorganism.

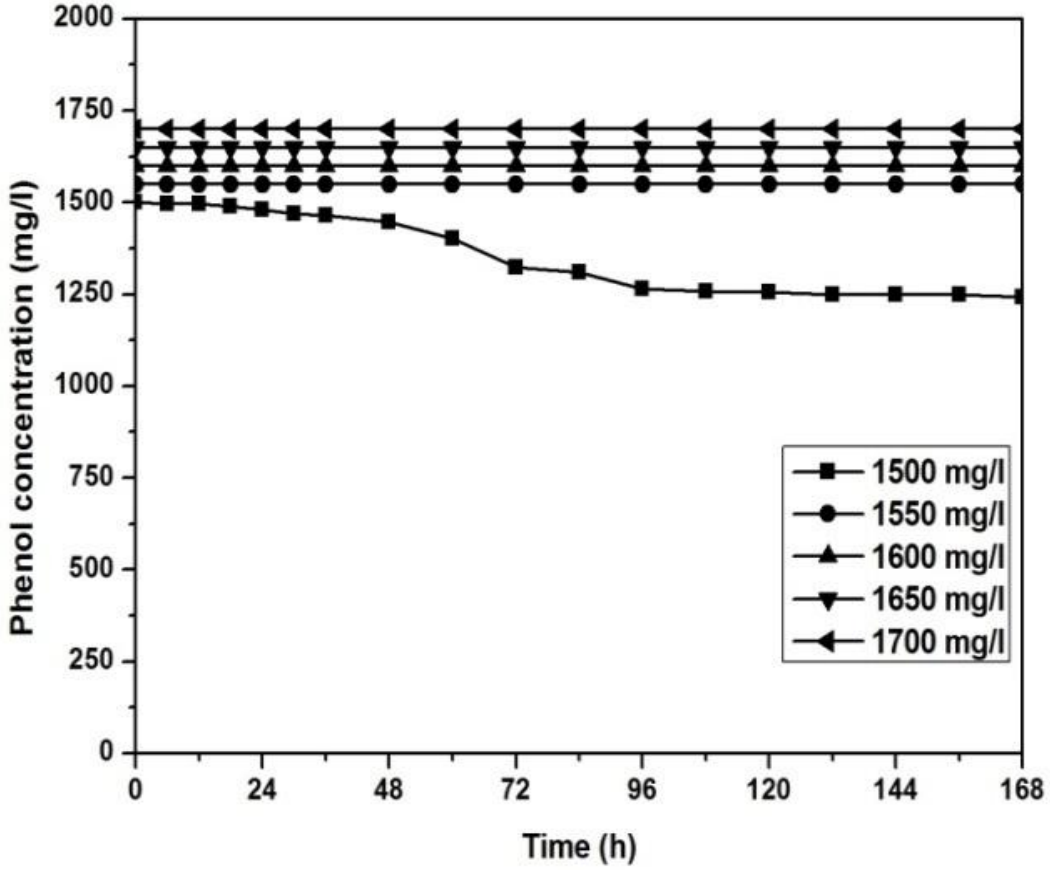


Fig.3.25. Phenol degradation profile for *Burkholderia sp*. PS3 at various initial higher phenol concentrations

3.5.2 Degradation study of phenol by *Bacillus pumilus* OS1:

Phenol degradation behavior of *Bacillus pumilus* OS1 has been studied at 500-1250 mg/l phenol concentration with the interval of 250 mg/l and 1250-1450 mg/l with the interval of 50 mg/l. The other parameters have been kept at their optimized level i.e. pH - 7.07, temperature 29.3°C, inoculum size - 6.3% (v/v) and $(NH_4)_2SO_4$ - 392.1 mg/l. Fig.3.26 depicts growth curve of *Bacillus pumilus* OS1 at various initial phenol concentrations. This figure shows that from 500 to 1250 mg/l of phenol, lag phase has been observed and its period has been increased as phenol concentration increased. As shown in Fig.3.27, at an initial phenol concentration of 1300 mg/l and above no microbial growth has been observed and thus it implies that the microbes can tolerate up to 1250 mg/l of phenol and above this concentration it gets completely inhibited.

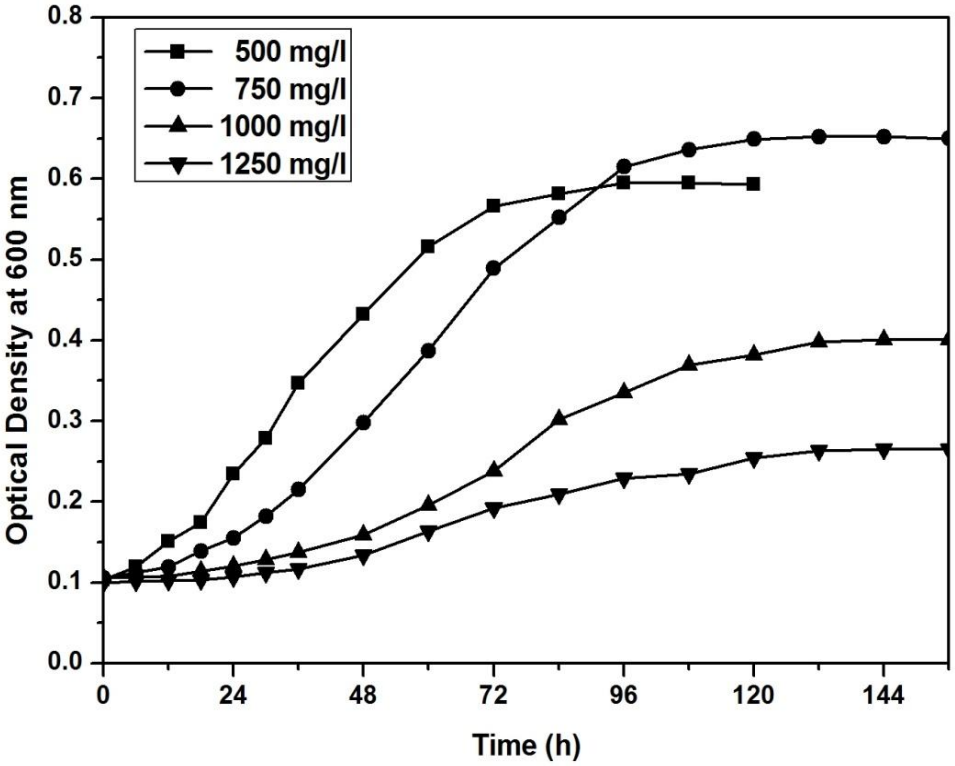


Fig.3.26. Growth profile for *Bacillus pumilus* OS1 at various initial phenol concentrations

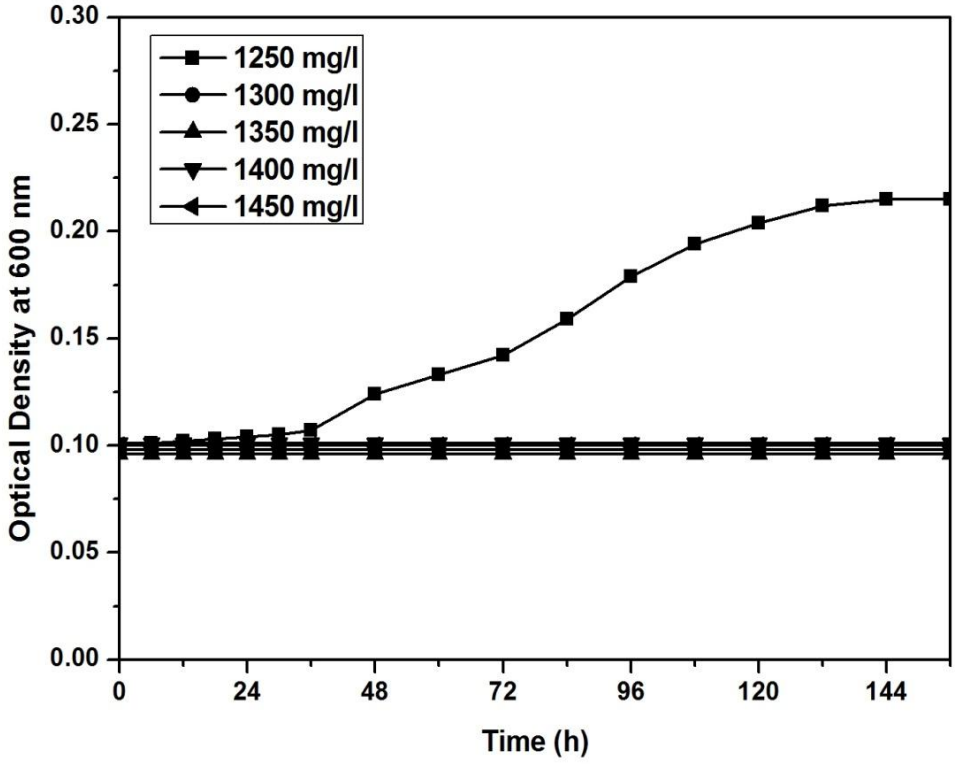


Fig.3.27. Growth profile for *Bacillus pumilus* OS1 at various initial higher phenol concentrations

Fig.3.28 represents phenol degradation profile for *Bacillus pumilus* OS1 at various initial phenol concentrations. The 97.72%, 94.14%, 68.5% and 28.32% degradation of 500, 750, 1000 and 1250 mg/l phenol concentrations has been achieved. Fig.3.29 shows Phenol degradation profile for *Bacillus pumilus* OS1 at various initial higher phenol concentrations. It showed phenol degradation upto 1250 mg/l and it has been completely inhibited from concentration 1300 mg/l. Gunther et al. (1995) reported cometabolic degradation of phenol and cresol by *Bacillus pumilus*. There are very few reports available for phenol degradation by *Bacillus pumilus* where phenol as a sole carbon source. Gayathri and Vasudevan (2010) reported 60% phenol removal efficiency at 300 mg/l concentration by *Bacillus pumilus* isolated from soil contaminated with industrial effluent. This shows that isolated *Bacillus pumilus* OS1 tolerates high phenol concentration than the previously reported strains.

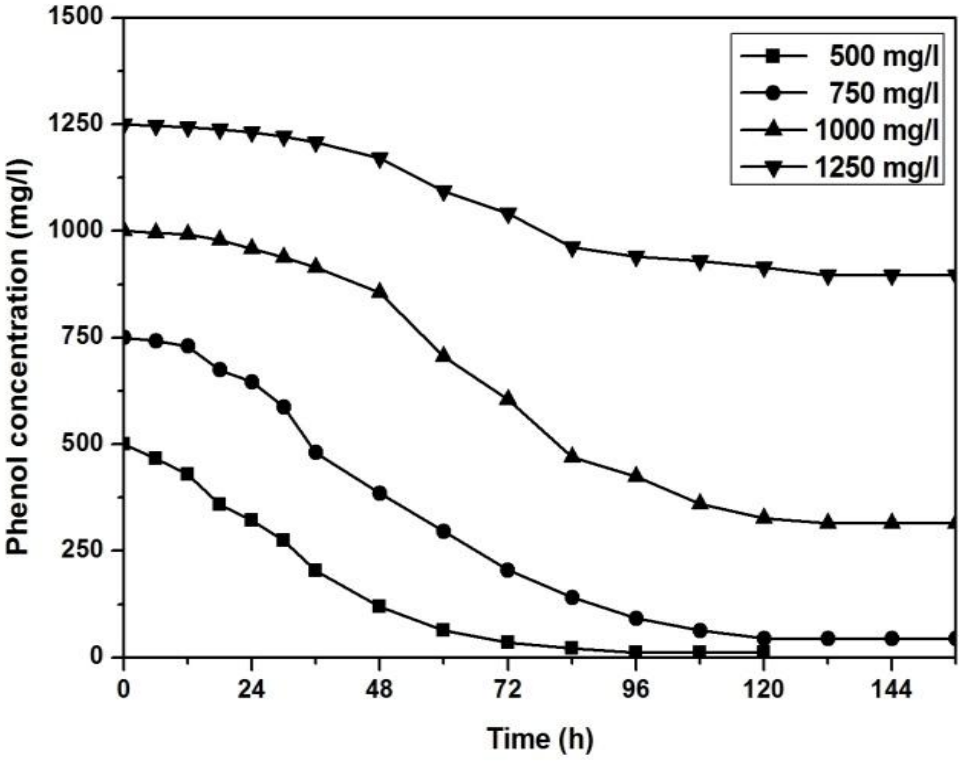


Fig.3.28. Phenol degradation profile for *Bacillus pumilus* OS1 at various initial phenol concentrations

For both the strains, it has been found that the phenol degradation rate reduced at the end of phenol degradation curve. This might be due to decrease in pH of the solution during the degradation period (Arutchelvan et al., 2006). The incomplete phenol degradation by both the isolated strains might be due to the presence of substrate inhibition and the toxicity of high phenol concentration (Bakhshi et al., 2011; Luo et al., 2009). Even if the isolated strains are not able to utilize the phenol completely, it is able to tolerate such

high concentration of phenol which makes them potential candidates in the field of phenol biodegradation.

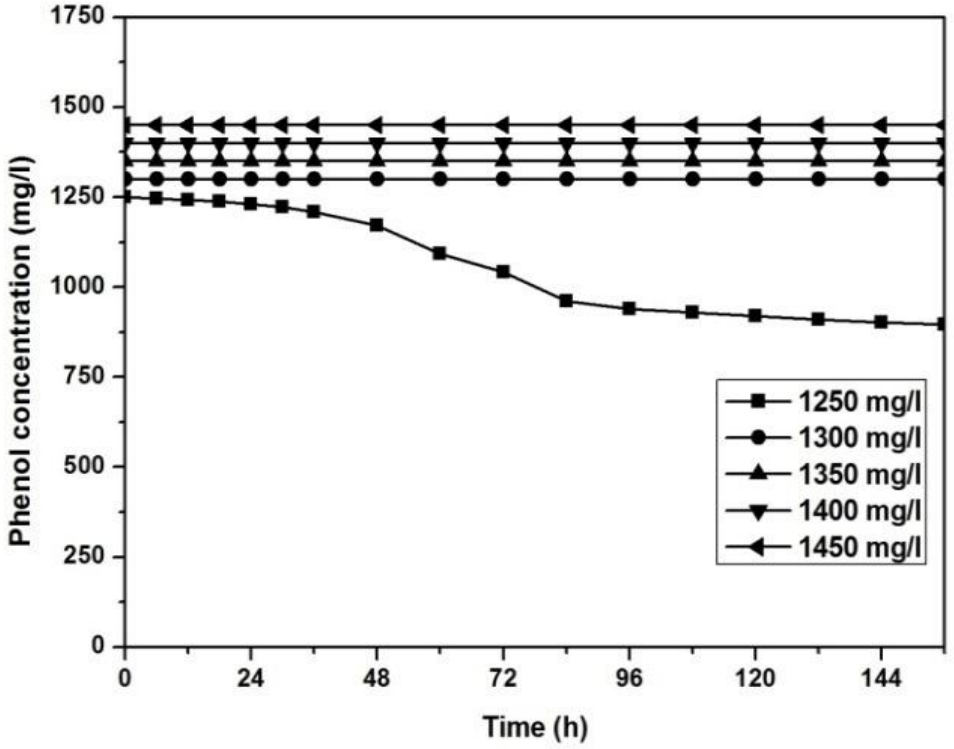


Fig.3.29. Phenol degradation profile for *Bacillus pumilus* OS1 at various initial higher phenol concentrations

As shown in Figs.3.19, 3.20, 3.23 and 3.27, the growth of both the strains has been enhanced at their optimized growth conditions. Similar result has been reported by Suhaila et al. (2013), that growth of *Rhodococcus* UKMP-5M has been increased at optimum conditions for phenol biodegradation. For both the strains, lag phase period has been increased as initial phenol concentration increased and the occurrence of lag phase at high phenol concentrations might be due to toxicity of phenol. Dey and Mukherjee (2010) and Saravanan et al. (2008) also described in their study that at high phenol concentrations, longer lag phase adopted as phenol is a growth limiting substrate.

3.6 Growth Kinetics of isolated strains for phenol biodegradation

Determinations of growth of microbes and growth kinetic parameters have high importance in biodegradation study. Haldane kinetic model has been proposed as the best model to indicate inhibition effect of phenol on microbes. In present study, the kinetic parameters for phenol degradation by isolated *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 have been obtained by fitting their respective experimental growth data to Haldane model equation (Equation 2.3). The maximum specific growth rate (μ_{max}), half-

saturation coefficient (K_s) and substrate inhibition constant (K_i) have been determined for different initial phenol concentration used for each strain.

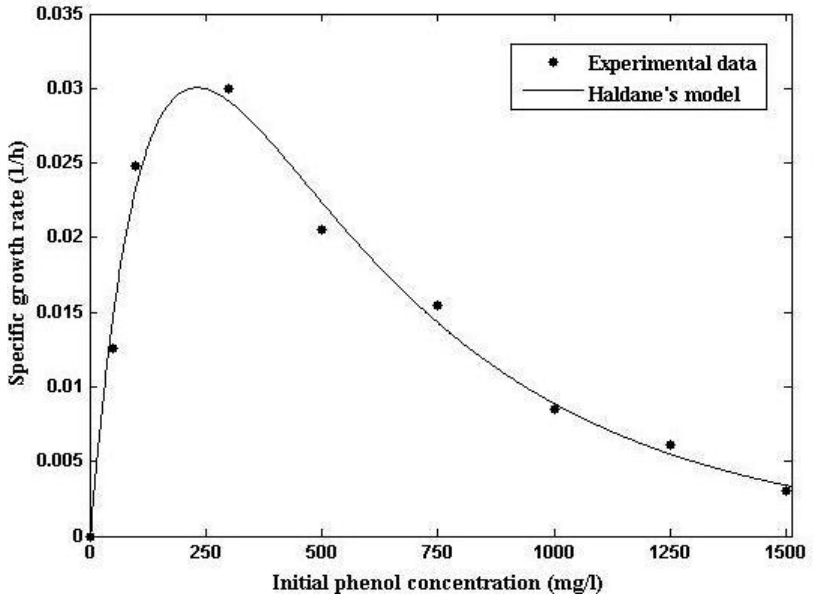


Fig.3.30. Haldane growth kinetic model fitted to experimental batch growth data of *Burkholderia sp*. PS3

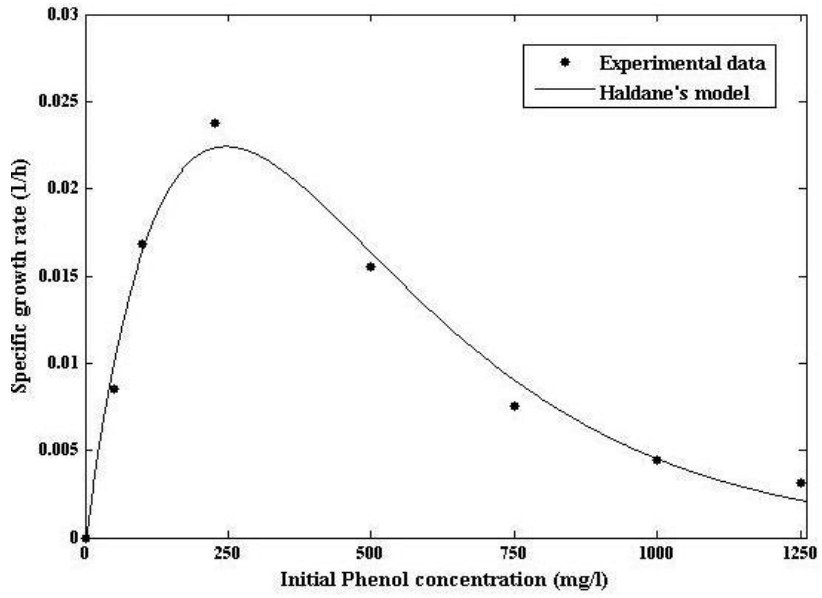


Fig.3.31. Haldane growth kinetic model fitted to experimental batch growth data of *Bacillus pumilus* OS1

Fig.3.30 and 3.31 show the experimentally obtained specific growth rates and specific growth rates predicted by model at various initial phenol concentrations for strains PS3 and OS1 respectively. The μ_{max}, Ks, and K_i for each initial phenol concentration used for *Burkholderia sp*. PS3 and *Bacillus pumilus* OS1 are enlisted in Table 3.14. Correlation

coefficient (R^2) found as 0.9845 and 0.9817 for strains PS3 and OS1 respectively, indicate experimental data is fit well for model. These figures shows that the value of specific growth rate increases with the increase in initial phenol concentration upto a optimum phenol concentration level, then it starts decreasing with further increase in the concentration. This indicates that phenol have inhibitory effect at high concentrations. Previously, Juang and Tsai (2006) and Bakhshi et al. (2011) studied kinetics of phenol degradation by using Haldane model and reported the inhibitory effect of phenol at high initial concentrations. Thus in present study the Haldane model explained well the inhibition effect of phenol at high concentrations.

Table 3.14: Haldane's growth kinetic parameters for phenol degradation by isolated strains

Strains	**Haldane Model**		
	μ_{max} (h^{-1})	**K_s (mg/l)**	**K_i (mg/l)**
Burkholderia sp. PS3	0.0436	29.43	839.90
Bacillus pumilus OS1	0.0370	38.27	587.62

3.7 Biodegradation of phenol by immobilized cells

Immobilization of cells is the effective phenolic effluent treatment as it enhances tolerance and efficiency at high phenol concentrations. The entrapment of cells in Ca-alginate has been a promising method for microbial degradation of phenol (Chung et al., 2003). Hence, the isolated *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 have been immobilized in calcium alginate beads and subsequently the independent experiments have been performed at various initial phenol concentrations. Phenol degradation behavior of immobilized *Burkholderia* sp. PS3 has been studied at 500-1500 mg/l phenol concentration with an interval of 250 mg/l and 1500-1700 mg/l with an interval of 50 mg/l. Fig. 3.32 represents the phenol degradation profile of immobilized *Burkholderia* sp. PS3 at 500-1500 mg/l phenol concentration. Phenol concentrations 500, 750 and 1000 mg/l have been completely degraded within 108, 156 and 228 h respectively. The 69.92% of 1250 mg/l and 31.27% of 1500 mg/l phenol degradation has been achieved and it has been 18.72% and 13.97% higher than that of free cells respectively. Fig.3.33 shows the degradation profile of immobilized sp. *Burkholderia* sp. PS3 at higher initial concentrations of phenol (1500-1700 mg/l). Immobilized *Burkholderia* sp. PS3 showed 21.2% and 11.93% degradation of 1550 and 1600 mg/l phenol, but it has been inhibited at a phenol concentration of 1650 mg/l and above.

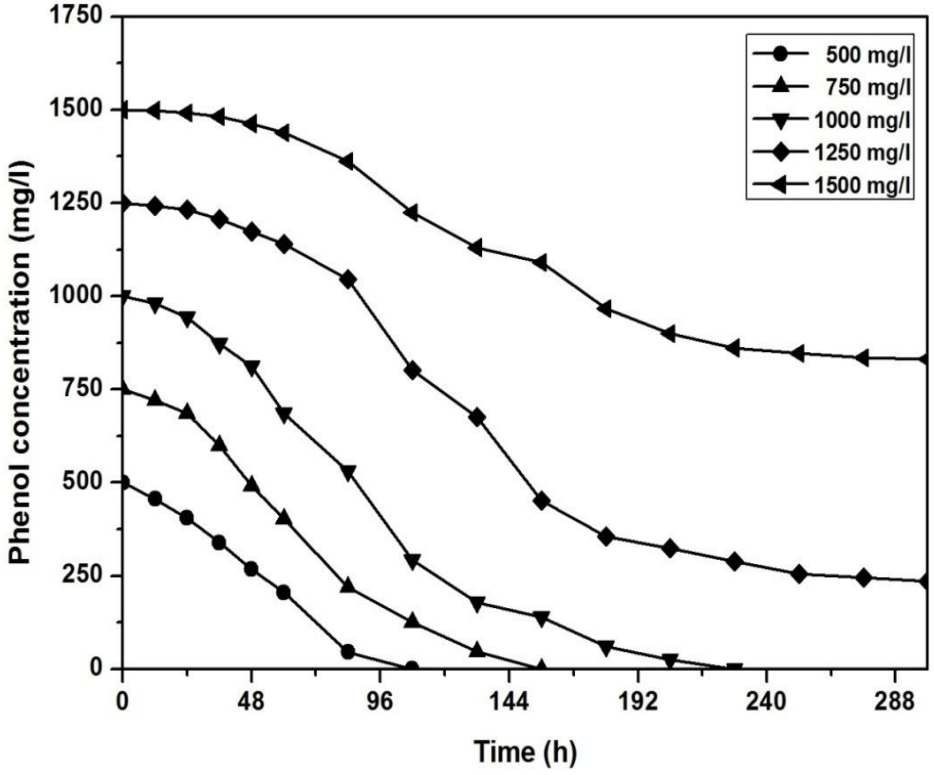


Fig.3.32. Phenol degradation profile of immobilized *Burkholderia sp*. PS3.

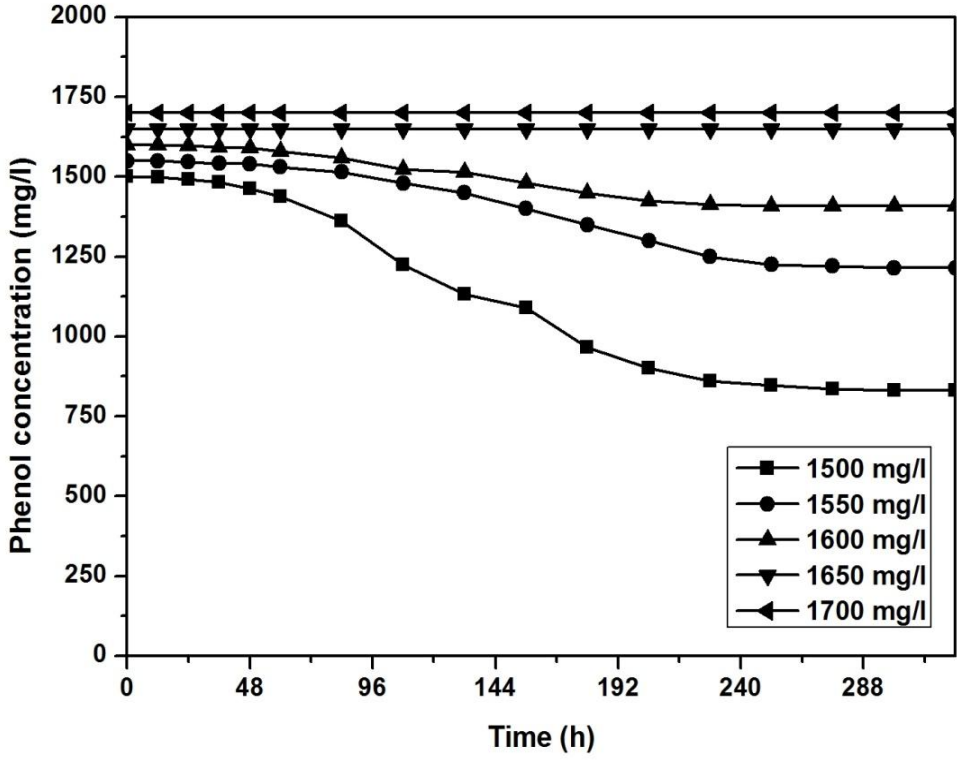


Fig.3.33. Phenol degradation profile of immobilized *Burkholderia* sp. PS3 at higher phenol concentrations.

Phenol degradation behavior of immobilized *Bacillus pumilus* OS1 has been studied at 500-1250 mg/l phenol concentration with the interval of 250 mg/l and 1250-1450 mg/l with the interval of 50 mg/l. As shown in Fig.3.34, phenol concentrations 500 and 750 mg/l have been completely degraded within 120 and 192 h respectively. The 92.4% of 1000 mg/l phenol has been degraded which is 23.9% higher than that for free cells while 46.88% of 1250 mg/l phenol has been degraded and it has been 17.76% higher than that of free cells. Fig. 3.35 shows the degradation profile of immobilized *Bacillus pumilus* OS1 at higher concentrations of phenol. Immobilized *Bacillus pumilus* OS1 showed 14.53% degradation of phenol concentration 1300 mg/l and it has been inhibited from 1350 mg/l phenol.

The possible reasons for slower degradation by immobilized cells as compared to free cells could be intraparticle diffusion limitation and limited space for cellular growth due to the gel–core structure (Yoo et al., 1996; Aksu and Bulbul, 1999; Chung et al., 2003). It has been found that for strain PS3 and strain OS1, the percentage degradation of phenol has been enhanced as compared to their respective free cells. Similarly, Banerjee and Ghoshal (2011) reported that the immobilization of cells in calcium alginate improved substrate tolerance and efficiency of biodegradation of phenol at higher phenol concentrations with slower degradation as compared to free cells. Park et al. (2013) also reported that, for phenol concentrations 50 – 500 mg/l, immobilized cells in calcium alginate showed slower degradation rate and at phenol 1000 mg/l, % degradation has been increased as compared to free cells. Dursun and Tepe (2005) found lower substrate removal rate values for immobilized cells in calcium alginate than free cell because of internal mass transfer limitations and they reported immobilized microorganism could expose to higher phenol concentration without loss of cell viability. Thus in present study immobilization of isolated strains in calcium alginate beads enhanced the efficiency of phenol degradation and tolerance at higher phenol concentrations.

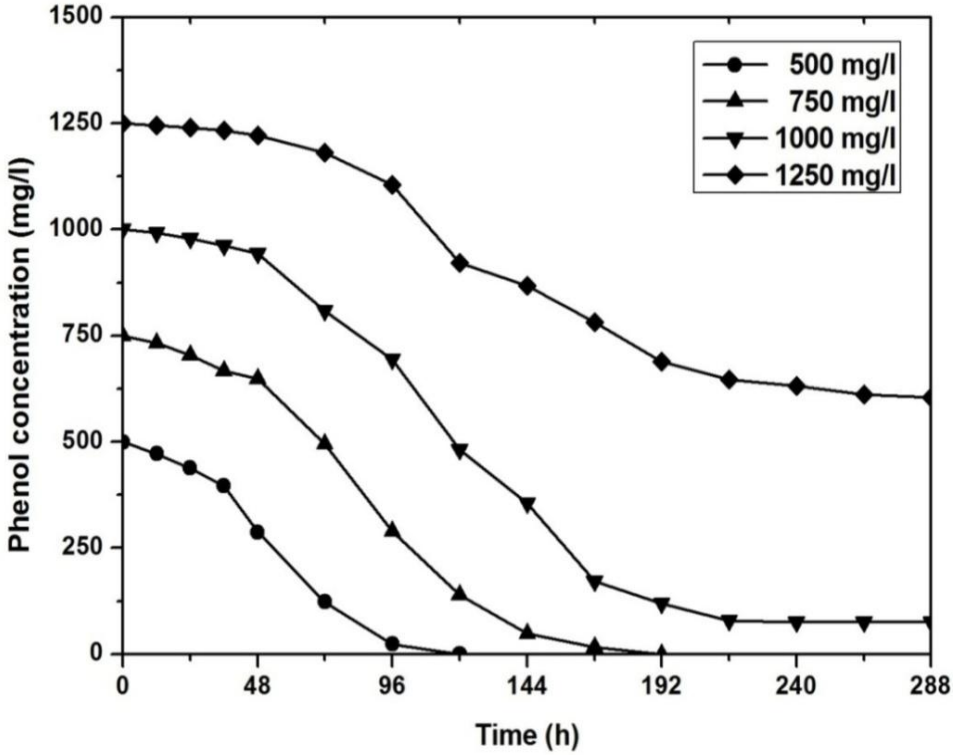


Fig.3.34. Phenol degradation profile of immobilized *Bacillus pumilus* OS1.

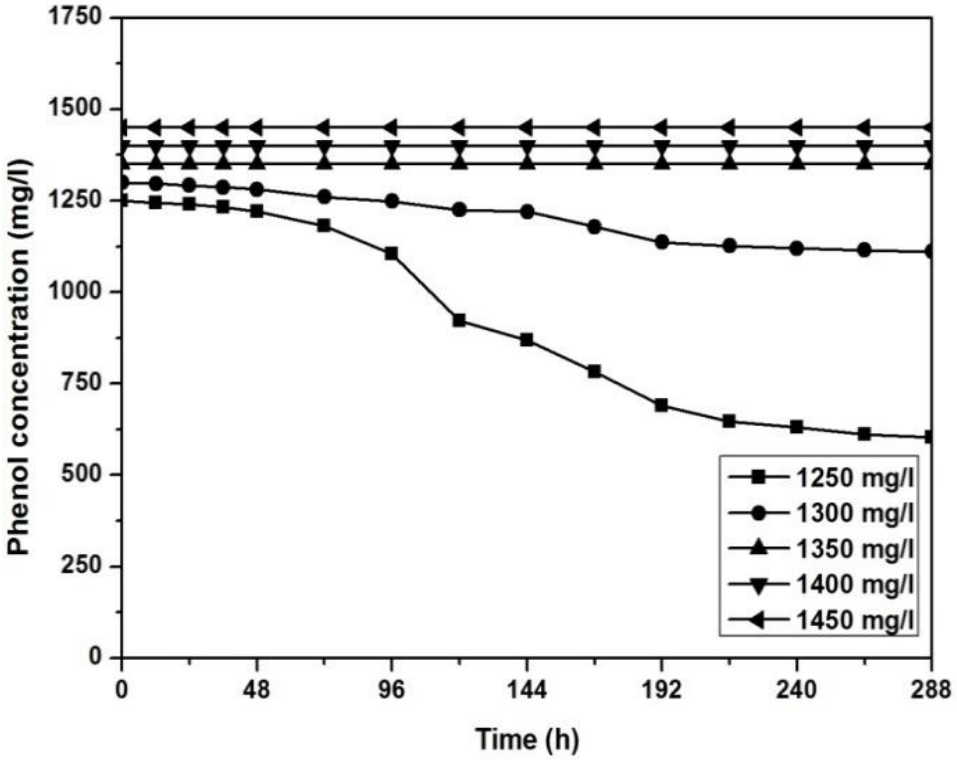


Fig.3.35 Phenol degradation profile of immobilized *Bacillus pumilus* OS1 at higher phenol concentrations.

CONCLUSION
AND
FUTURE WORK

Chapter-4

Conclusion and Future work

In the present work an attempt has been made to isolate, identify and characterize highly efficient phenol degrading bacterial strains from soils contaminated with paper mill effluent and crude oil. The points have been concluded are bulleted as follows:

- Two highly efficient bacterial strains; PS3 tolerating phenol concentration up to 1500 mg/l and strain OS1 tolerating phenol concentration up to 1250 mg/l have been isolated from soil contaminated with paper mill effluent and crude oil respectively.
- On the basis of morphological, biochemical and molecular characteristics, strain PS3 has been identified as *Burkholderia* sp. and strain OS1 has been identified as *Bacillus pumilus*.
- The 16S rRNA partial gene sequences of both the strains have been registered in Nucleotide database of NCBI with GenBank accession numbers KJ530761 and KJ530762 respectively.
- From detailed literature review, a total of nine independent variables: pH, temperature, inoculum size and concentrations of phenol, KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, NaCl and $MgSO_4$ have been identified to effect the phenol degradation. These parameters (variables) along with two dummy variables have been screened using for both the isolated strains independently.
- By using Plackett-Burman design, the parameters; pH, temperature, phenol concentration and inoculum size have been found to be significant for phenol degradation by *Burkholderia* sp. PS3. Whereas parameters; pH, temperature, phenol concentration, inoculum size and $(NH_4)_2SO_4$ concentration have been found to be significant for phenol degradation by *Bacillus pumilus* OS1.
- The optimum levels of significant factors have been identified by using central composite design (CCD). The maximum phenol degradation of 99.96% by *Burkholderia* sp. PS3 has been predicted at pH - 7.18, temperature - 28.9°C, phenol - 297.9 mg/l and inoculum size - 5.04% (v/v). Under these conditions, 99.88% phenol degradation has been achieved by validating experiment which is very close to the predicted value.
- For *Bacillus pumilus* OS1, a maximum phenol degradation of 99.99% has been predicted at pH - 7.07, temperature - 29.3°C, phenol - 227.4 mg/l, inoculum size -

6.3% (v/v) and $(NH_4)_2SO_4$ - 392.1 mg/l. The validating experimental result of 99.90% is significant agreement the predicted ones.

- The close agreement between predicted and experimental results demonstrates the accuracy of the models in predicting the optimum conditions. Thus by using these models it is possible to determine the response (phenol degradation) for different values of the parameters.
- The duration of lag phase has been found to increase due to inhibition effect of phenol at higher initial phenol concentrations. At phenol concentrations of 500, 750, 1000, 1250 and 1500 mg/l, a degradation of 98.86%, 98.62%, 83.3%, 51.2% and 17.3% has been achieved by *Burkholderia* sp. PS3 respectively. *Bacillus pumilus* OS1 has shown 97.72%, 94.14%, 68.5% and 28.32% degradation at 500, 750, 1000 and 1250 mg/l of phenol concentrations respectively. Due to toxicity the final degradation has been reduced as the initial phenol concentration is increased for both the isolated strains.
- Haldane model has been found to fit well to the experimental growth data observed for the microbes with coefficient of correlation (R^2) of 0.9845 and 0.9817 for *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 respectively.
- The growth kinetic parameters; μ_{max}, maximum specific growth rate; K_s, half-saturation coefficient and K_i, the substrate inhibition constant have been evaluated for both the strains independently. For *Burkholderia* sp. PS3, μ_{max} = 0.0436 h-1, K_s = 29.43 mg/l and K_i = 839.90 mg/l while for *Bacillus pumilus* OS1, μ_{max} = 0.0370 h-1, K_s = 38.27 mg/l and K_i = 587.62 mg/l have been estimated. The values of specific growth rate show that the increase in phenol concentration decreases the growth rate which suggests the toxic nature of the phenol.
- The immobilized cells of *Burkholderia* sp. PS3 and *Bacillus pumilus* OS1 has shown complete phenol degradation up to 1000 mg/l and 750 mg/l respectively.
- Immobilized *Burkholderia* sp. PS3 is able to degrade 69.92% and 31.27% of phenol at 1250 mg/l and 1500 mg/l of phenol concentration, which are 18.72% and 13.97% higher than that degraded by free cells respectively under the same condition.
- Immobilized *Bacillus pumilus* OS1 has shown 92.4% and 46.88% of phenol degradation at concentration of 1000 mg/l and 1250 mg/l phenol, which is 23.9% and 17.76% higher than that, achieved for free cells respectively.

- Immobilized *Burkholderia* sp. PS3 has found to tolerate 1600 mg/l of phenol while immobilized *Bacillus pumilus* OS1 has shown tolerance up to 1350 mg/l of phenol.
- As compared to free cells, immobilized cells have shown better tolerance and higher phenol degradation efficiency at high concentrations. These strains are potential candidates for phenol degradation.

Future work:

The followings are the recommendation for the future work.

- Parameters optimization of immobilized cells for maximal phenol degradation.
- Study of various low cost effective materials for immobilization of strains and subsequent biodegradation study of phenol by immobilized cells.
- Determination metabolic pathway of phenol degradation for isolated strains.
- Large scale treatment of phenolic effluent by the isolated strains.
- Characterization of the enhancement of tolerance and degradation efficiency of the isolated microbes for its use in immobilized cell bioreactors.

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Appendix-A

Standard Curve for Biomass weight:

The biomass samples have been dried in Hot air oven at 80^{o}C and the difference between initial and final weight has been taken as a result. For *Burkholderia* sp. PS3 (Fig.1) and *Bacillus pumilus* OS1 (Fig.2), the standard curves have been found linear with R^2= 0.9996 and R^2= 0.9993 respectively.

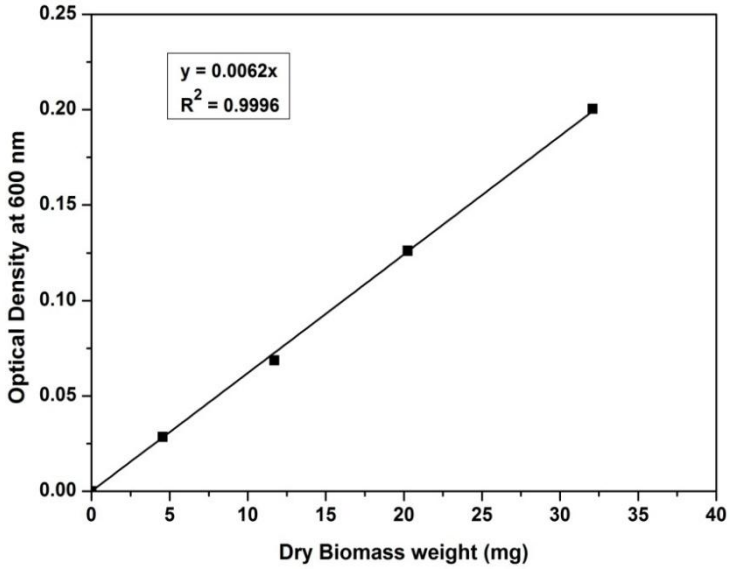

Fig.1. Standard Curve for Biomass for *Burkholderia* sp. PS3

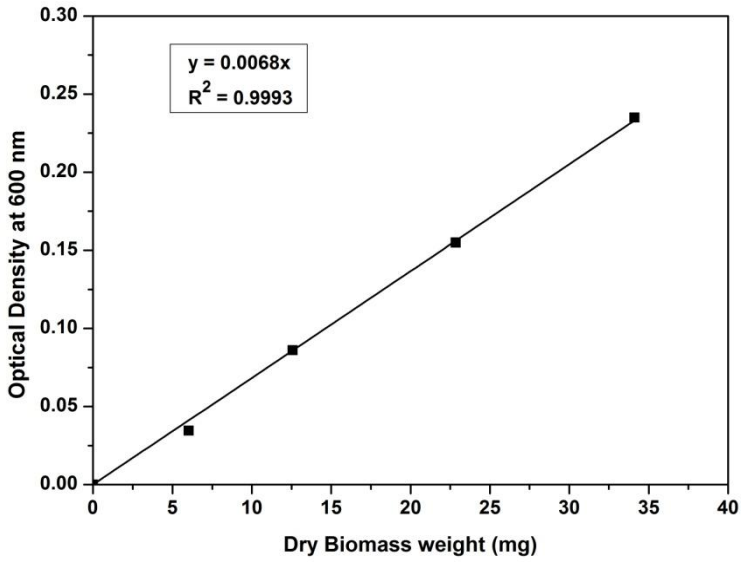

Fig.2. Standard Curve for Biomass for *Bacillus pumilus* OS1

Calibration Curve for standard phenol concentrations:

The standard phenol solutions (1-10 mg/l) have been used to prepare calibration curve. It has been found linear with R^2= 0.9965 (Fig.3).

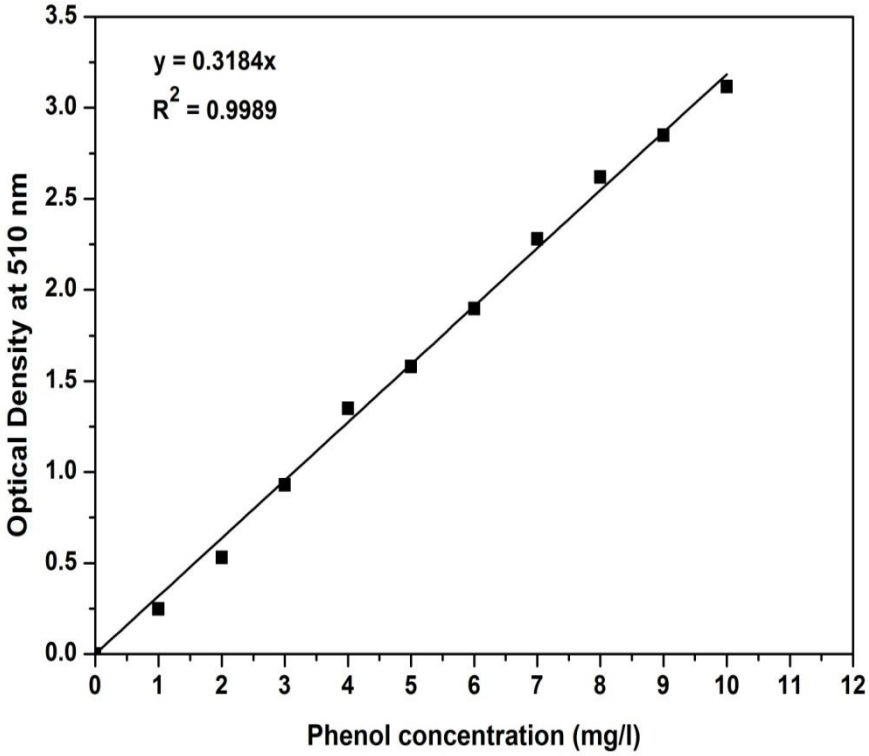

Fig.3. Calibration Curve for standard phenol concentrations

Appendix-B

Nutrient Agar Medium

Ingredient	Amount
Beef Extract	10g
Bacterial peptone	10g
Sodium Chloride	5g
Bacterial Agar	20g
Distilled Water	1000 ml

Nitrate Broth

Ingredient	Amount
Peptone	5g
Meat Extract	3g
Potassium Nitrate	1g
Distilled Water	1000ml

Nitrate reduction medium

Ingredient	Amount
Beef (meat) extract	3g
Gelatin peptone	5g
Potassium nitrate	1g
Distilled Water	1000ml
Reagent A	
N, N-Dimethyl-α-naphthylamine	0.6 ml
Acetic acid (5N)	100 ml
Reagent B	
Sulfanilic acid	0.8 g
Acetic acid (5N)	100 ml

Reagents A and B have been stored in the refrigerator.

Phenol red carbohydrate broth

Ingredient	Amount
Peptone	10 g
Sodium chloride	5 g
Beef extract	1 g
Phenol red	0.018 g
(7.2 ml of 0.25% phenol red solution)	
Distilled water	1000 ml
Carbohydrate to be tested	10 g

Christensen's Urea Agar

Ingredient	Amount
Peptone	1 g
Dextrose	1 g
Sodium chloride	5 g
Potassium phosphate, monobasic	2 g
Urea	20 g
Phenol red	0.012 g
Agar	15 g

Gelatin Agar

Ingredient	Amount
Peptone	5 g
Beef extract	3 g
Gelatin	4g
Agar	18g
Distilled water	1000 ml
pH	7.0

Starch agar

Ingredient	Amount
Beef extract	3 g
Soluble starch	10 g
Agar	12 g
Distilled water	1000 ml

Tryptone broth

Ingredient	Amount
Tryptone	10 g
Sodium chloride	5 g

MR-VP Medium

Ingredient	Amount
Peptone	7g
Dextrose	5g
Dipotassium Phosphate	5g
Double distilled water	1000ml
pH	6.9 ± 0.2

Vogues Proskauer reagents

Barritt's reagent A: 5% (wt/vol) a-naphthol in absolute ethanol
Barritt's reagent B: 40% (wt/vol) KOH in distilled water

Simmons citrate medium

Ingredient	Amount
Magnesium sulfate (heptahydrate)	0.2 g
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium citrate (dehydrate)	2 g
Sodium chloride	5 g
Agar	15 g
Bromothymol blue	0.08 g
Distilled water	1000 ml
pH	6.9

Preparation of Phosphate buffer (pH: 7.4)

- 80.2 ml of 1M K_2HPO_4 has been added to 19.8 ml of 1M KH_2PO_4, such that final pH would be 7.4

Preparation of 0.5X TAE (pH 8.0) buffer

- 600 ml of Milli-Q water has been added to 242 g tris, 57.1 ml acetic acid and 100 ml 0.5M EDTA. The solution has been stirred and pH adjusted to 8. Total volume has been made to 1 litre.
- Buffer has been stored at room temperature.

Preparation of Kovac's Reagent:

- 25 ml of conc. HCL has been added to75 ml of Amyl alcohol.
- 5g of 4-Dimethylaminobenzenealdehyde has been dissolved in the solution.
- The reagent has been stored in refrigerator in brown glass bottle.

Preparation of Glycerol Stock:

- The bacterial cultures have been incubated overnight for preparation of the glycerol stock.
- 700µL of the overnight grown culture has been added to 300 µL of autoclaved glycerol and mixed properly and has been immediately transferred to ice.
- It has been stored at -20°C for future use.

Preparation of TE Buffer:

- 100mM of Tris HCL (pH-8.0)+10mM EDTA(pH-8.0)
- To 1.21g of Tris Cl has been dissolved with 0.372g of EDTA and the volume has been made
  up to 100ml after adjusting the pH 8.0.

Preparation of CTAB-NaCl Solution:

- To 4.1g of NaCl, 80ml of water and 10g of CTAB has been added to it while heating and stirring continuously. It has been heated up to 65oC to dissolve and later the volume has been adjusted to 100ml.

Preparation of Tris Saturated Phenol:

- Phenol has been melted at 68oC and hydroquinoline has been added to a final concentration of 0.1%.
- Equal volume of 1M Tris-Cl has been added and the mixture has been added for 15 minutes.
- The upper aqueous layer has been removed.
- The above two steps have been repeated with the lower layers with 1M Tris (pH-8.6) and finally with 0.5M Tris (pH-8.6) for 2-3 times until the pH of the phenol reaches 8.0.
- 0.1M Tris (equal volume) of pH 8.0 has been added to phenol containing 0.2% β-mercaptoethanol and stored in dark amber colored bottle at 4^{o}C.

Appendix-C

Estimation of Phenol:

Reagents:

***Ammonium hydroxide* (0.5N):** 35 ml fresh, concentrated NH_4OH has been diluted to 1 l with water.

Phosphate buffer solution: 104.5 g K_2HPO_4 and 72.3 g KH_2PO_4 has been dissolved in water and diluted to 1 l. The pH has been adjusted to 6.8.

4-Aminoantipyrine solution: 2.0 g 4-aminoantipyrine has been dissolved in water and diluted to
100 ml.

Potassium ferricyanide solution: 8.0 g $K_3Fe(CN)_6$ has been dissolved in water and diluted to 100 ml. The solution has been stored in a brown glass bottle and prepared fresh weekly.

Procedure:

- 100 ml of sample has been taken and 2.5 ml 0.5N NH_4OH solution has been added and pH has been immediately adjusted to 7.9 ± 0.1 with phosphate buffer.
- 1 ml 4-aminoantipyrine solution has been added and mixed well,
- 1 ml $K_3Fe(CN)_6$ solution has been added and mixed well.
- After 15 min, absorbance of sample and standards has been taken against the blank at 500 nm.

Appendix-D

Table 1: List of Instruments used during the experimental study

Instrument	**Make**
Analytical Balance	Contech
pH Meter	Systronics
Vertical Autoclave	Reico
Laminar air flow chamber	Zhichen (ZhJH-1109C)
Bacteriological Incubator	Incon
Spectrophotometer (UV/Vis)	Jasco (V-530)
Incubator shaker	Incon
Scanning Electron Microscope	JEOL (JSM, Japan)
Optical microscope	Hund (H-600)
Micro Centrifuge	Remi (CM-12 plus
Double distillation column	Borosil
Horizontal Gel electrophoresis	Bio-Rad (SubCell® 96)
PCR (Thermal Cycler)	Applied Biosystems (VeritiTM)
DNA sequence analyzer(Genetic analyzer)	Applied Biosystems (3730 xl)

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List of Paper Communicated:

- **Patil, S. S.**, Jena, H.M., 2014. Statistical optimization of phenol degradation by *Bacillus pumilus* OS1 using Plackett-Burman design and response surface methodology. Bioresource Technology.
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