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Screening and evaluation of phenol utilization and growth in *Acinetobacter baumannii* W29 of wastewater

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Abstract

Phenols are ubiquitous pollutants, mainly from industrial effluent, causing pollution of natural water resources. The research focused on screening efficient phenol-degrading bacteria and kinetic modelling of phenol biodegradation and growth. Membrane filtration was used for the isolation of bacteria from the wastewater sample. The screening of phenol-degrading bacteria was based on the efficiency of phenol utilization. The strain with efficient phenol degradation capacity was characterized by 16S rDNA sequencing and designated *Acinetobacter baumannii* W29. Biomass growth and phenol utilization rate of the strain were evaluated at different initial phenol concentrations (100-800 mgL^{-1}). Specific growth rate data were fitted to five models, i.e. Monod, Haldane, Aiba, Teisser, and Webb model. The yield coefficient at different initial phenol concentrations was calculated from the slope of the specific growth rate (μ) versus the specific phenol utilization rate (q). The strain showed complete phenol degradation potential up to 1000 mgL^{-1}. The maximal growth rate was achieved at 400 mgL^{-1} , which coincided with the maximum substrate utilization rate at the same concentration. The specific growth rate showed the best fit with the Haldane model. The strain had a yield coefficient of 0.70 (mg cell mg^{-1} phenol). The value of μ and K_s revealed the affinity of the strain for high-concentration phenol and the its ability to withstand high phenol concentrations. The kinetic growth behaviour of the strain fitted well with the Haldane model. The findings of the study could be applied to wastewater treatment with a high phenol load.

Keywords: *Acinetobacter baumanii* W29**,** Batch culture, Phenol utilization, Screening, 16S rRNA gene sequencing, Kinetic analysis

INTRODUCTION

With the huge rise in wastewater toxic and recalcitrant chemicals, there is growing concern about developing new advanced, effective remediation and environmentally sustainable technologies to tackle water contamination. Industrial wastewater discharges, such as petrochemical, tannery, coke wastewater, pulp and paper contain high phenolic concentrations (Villegas *et al*., 2016). Phenol, a protoplasmic toxin causes cell membrane destruction, protein denaturation, and cytoplasmic coagulation leading to cell death and necrosis (Downs and Wills, 2020). It has been shown to affect the metabolism, survival, and reproductive ability of fish, while sub-lethal doses of phenol interact with various enzyme activities (Al-Khalid and El-Naas, 2012).

It is reported that phenol has a low level of persistence in air, water, and soil has a low bioaccumulation potential and is moderately harmful to aquatic species (Bingham and Coherssen, 2012). However, phenol

contamination of underground water has been reported to persist for several years. Phenol toxicity may disrupt the aquatic food web and affect higher food chains, such as humans, by affecting the organs, especially the nervous system (Duan *et al.*, 2018). Several environmental bodies around the world, such as the World Health Organization (WHO), Environmental Protection Agency (EPA), USA and the European Union (EU), have listed phenol as a priority pollutant. A high concentration of phenol, as high as 3000 mgL^{-1} in industrial effluents, is reported (Hussain *et al.*, 2015). In India, 0.001 mgL^{-1} of phenolic compounds is acceptable in drinking water, and 0.002 mgL^{-1} is the permissible limit as per BIS: 10500 (1991). Therefore, the concentration of phenolic pollutants in wastewater must be brought within acceptable limits through pre-treatment processes before release into the environment.

Various separation technologies, such as nano-filtration, distillation, absorption, extraction, etc. and destruction technologies such as oxidation, ozonolysis, electrochemical and photocatalytic oxidation, etc. have been practiced removing phenol. However, these approaches have many drawbacks, such as the complexity of the process, toxic by-product production, inability to degrade low concentrations of phenol, high operating costs, and high energy consumption. Contrary to it, biological degradation of phenol has been considered an alternative method with complete mineralization potential, no toxic by-product formation, and can be operated relatively cheaply.

Many microorganisms are capable of using phenol as the primary source of carbon and energy and several efficient strains were isolated from high-phenolic polluted environments, such as *Psuedomonas* (Wasi *et al.*, 2013), *Acinetobacter* (Liu *et al.*, 2016) and *Candida tropicalis* (e Silva *et al.*, 2019). The literature review suggests that Acinetobacter species can degrade high concentrations of phenol~ as 2000 mgL^{-1} (Yadzir *et al.*, 2016). The behaviour of high-strength phenol degrader under low phenol micro pollution conditions is less well understood.

Aerobic degradation is typically achieved via ortho or meta pathways, leading to the formation of cis, cis-muconic acid/ or 2-hydroxymuconic semialdehyde (2-HMSA), respectively, which are further metabolized in the tricarboxylic acid cycle (TCA). The biochemical degradation pathways and kinetic mechanisms may differ under a low/or high phenolic environment. With the change of phenol concentration from low to high, change in the degradation pathway as well as biodegradation kinetics has been reported (Lim *et al.*, 2013). To date, little information is available in the literature on the kinetic response of high phenol degraders such as *Acinetobacter baumannii*. Therefore, the present study focused on investigating the kinetic behaviour of growth and phenol degradation of *A. baumannii* W29.

MATERIALS AND METHODS

Media
The minimal salt media (MSM) with constituents KH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $CaCl_2$ 0.1 g, NaCl 0.2 g, $MgSO_4.7 H_2O$ 0.5 g, $MnSO_4.7 H_2O$ 0.01 g, $FeSO_4.7 H_2O$ 0.01 g, NH_4NO_3 1.0 g per liter and pH 7.2 was used for the preparation of liquid medium. The 15 g agar was added to the medium to obtain a semi-solid MSM medium.

Sample collection and isolation
Wastewater samples were collected from the Bindal river, Dehradun, Uttarakhand (India). The samples were taken by completely inverting the sample container over and immersing it 0.3 m under the water's surface.A total of 2L of wastewater was collected from the Bindal river, Dehradun (Uttarakhand) in a pre-sterile glass bottle. Then, samples were transported to the laboratory on ice packs within 1 h of collection. The viable count of the bacteria in the collected sample was $\sim 10^5$ CFU/ml. Isolation of bacteria from the wastewater sample was done by filtering water through the membrane filter paper as per Hamner *et al.* (2007) procedure with some modifications. Firstly, the wastewater was filtered under vacuum through Whatman number 1 filter paper. The filtrate was then passed into a 0.45μm pore membrane filter at the top of the 0.2μm membrane.

Screening phenol-tolerant bacterial strain
After membrane filtration as above, the bacteria contained on the membrane filter were cut into pieces, which were then immersed in the liquid MSM medium with phenol (5mgL^{-1}), then agitated in a rotary shaker at 100 rpm at 30^{0}C. The culture, showing signs of growth, as seen from turbidity, was again inoculated into freshly prepared MSM medium with phenol to acclimatize the strain to grow in the presence of phenol. After 21 days of acclimatization, the culture was inoculated to fresh MSM medium with phenol. The pure strain was isolated by performing serial dilution, pouring onto MSM agar, and picking up single colonies distinctly differing in morphology. The single colony was repeatedly streaked on an agar plate to isolate the pure strain and was grown in MSM medium with only phenol (100-1000 mgL^{-1}) as an energy source. The phenol degrading ability of strains was selected based on efficient phenol utilization capacity that was determined by measuring the remaining phenol in the medium. The most efficient phenol degrading strain was designated as W29.

Morpho-biochemical identification of strain
The phenol degrading strain was examined by Gram staining and characterized by shape. Different biochemical tests were conducted to identify the selected

phenol-degrading strain W29 according to Bergey's Manual (Holt *et al*., 1994).

Molecular characterization of strain

Molecular identification of strain W29 was performed by partial amplification of specific parts of 16S rDNA using universal bacterial primers (27F and 1492R). A fast DNA isolation kit from Q Biogene was used for gDNA. The PCR reaction mixture (30 µl) consisted of primers, 27F and 1492R universal bacterial primers (1µl each of 10 pmolµl^{-1}), gDNA (2.5µl), Taq polymerase (0.4 µl of 5Uµl^{-1}), dNTPs (0.6µl of 10 mM) and MgCl2 (1.8 µl of 25 mM), 3µl of 10X buffer and 19.7µl of distilled water. The amplified product was separated by agarose gel electrophoresis, gel previously cast with ethidium bromide (5µgml^{-1}). DNA band was visualized in UV light. The amplification product was sequenced using Big Dye Terminator. The sequence was submitted to National Center for Biotechnology Information (NCBI), and accession number\ was assigned to the isolated strain *Acinetobacter baumannii* W29 by Gene Bank as KF686823. The Basic Local Alignment Search Tool-nucleotides(BLASTn) program was used to align the partial rRNA of our strain to rRNA sequences in the NCBI database. The neighbour-joining method was used for building phylogenetic trees (Saitou and Nei, 1987).

Batch culture and biomass growth

An aliquot of 1ml of suspension culture (absorbance A600 nm 0.03-0.05) of phenol tolerant strain, *A. baumannii* W29 was inoculated in 100 ml of MSM medium containing phenol (100 mgL^{-1}to 800 mgL^{-1}) as an only energy source. The biomass concentration was measured at regular intervals by centrifuging 1 ml of suspension culture at 5000 rpm for 10 min, collecting and drying the pellet. Then, a calibration plot was made by plotting the dry mass of cells versus absorbance A600 nm. The specific growth (µ) and substrate utilization (q) rates were determined.

Determination of phenol degradation

For phenol concentration measurement, 2 ml suspension culture aliquots were removed from each batch culture at regular intervals and centrifuged at 6000 rpm for 10 min. The residual concentration of phenol in the supernatant was then determined using a 4- aminoantipyrine at 510 nm (APHA, 2017).

Substrate utilization rate

The substrate utilization (q) rates were determined following the procedure of Dey and Mukherjee (2010).

Kinetic modelling

Kinetic studies on substrate growth inhibition were conducted on *A. baumannii* W29 with phenol as the only energy source. Specific growth rate (µ) data at different substrate concentrations were fitted with classical kinetic models, namely Monod, Haldane, Aiba, Teisser, and Webb model. The nonlinear regression analysis was performed using a nonlinear curve fitting tool of MATLAB 7.0 software to determine the various kinetic parameters. The high value of the correlation coefficient (R^2) and the low value of the root mean square error (RMSE) indicated a better fit.

RESULTS AND DISCUSSION

A potential strain that effectively degrades toxic/ or xenobiotic compounds ata rapid rate is required in a microbial treatment of domestic / or wastewater. Some investigators have used a direct isolation method containing phenol to screen phenol degrading bacteria (Filipowicz *et al*., 2017**).** However, some microorganisms can not adapt and survive a high phenol concentration in the direct screening method.Therefore, it is necessary to adapt the bacteria in the presence of phenol to facilitate the induction of phenol-degradative enzymes.The adapted bacteria can degrade phenol at concentrations higher than their natural environment (Stoilova *et al*., 2017) due to the inducible synthesis of phenol-degrading enzymes. However, the phenol enrichment method has been typically used to isolate phenol degrading bacteria (Gu *et al*., 2016). Similarly, the enrichment and acclimatization method was used for the isolation of phenol-degrading bacteria. Throughout the present study, the screening of degrading phenol bacteria was performed by calculating biomass growth and phenol utilization rate when the strain was fed on phenol as the only energy source. The strain W29 exhibited efficient biomass growth and phenol utilization capacity.

The 16S rRNA gene is a molecular marker for the identification of bacterial species (Srinivasan *et al*., 2015). The strain was identified with the polyphasic approach, including partial sequencing of 16S rDNA and phylogenetic and morpho-biochemical analysis. The strain appeared to be Gram-negative coccobacilli, non-motile, aerobic, non-fermentative, xylose fermentative with acid production, catalase positive, and oxidase negative, which was presumptively classified as *Acinetobacter* sp. The 16S rRNA gene amplification by polymerase chain reaction resulted in a 1.5 kb amplicon (Fig.1). The partial 16SrDNA gene sequence of the strain W29 was deposited in NCBI and obtained accession number KF686823. BLASTn analyses partial 16S rDNA gene sequence showed a strong homology (99% sequence identity) with *A. baumannii* in the NCBI database. Henceforth, the strain was designated as *A. baumannii W29*. Phylogenetic analysis was carried out using the corrected distance model from Jukes-Cantor and the phylogenetic trees was generated using NJ
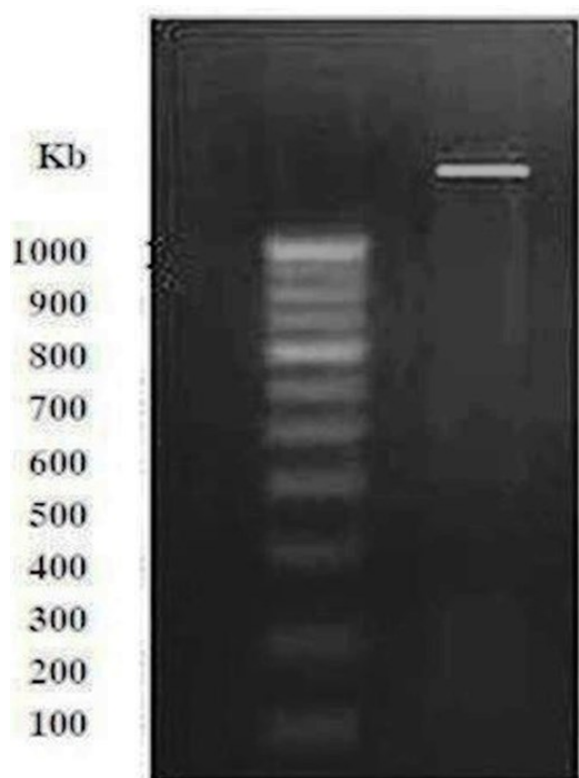
Fig. 1. *PCR amplification of16S rDNA of Acinetobacter sp. W29*

(Neighbor-Joining) method (Fig. 2.)

High phenol tolerance shown by different microorganisms is shown in Table1 for comparison with the strain under study, *A. baumanii* W29, which could completely degrade phenol 800 mgL^{-1} in 20 h. The growth of microorganisms corresponds to the degradation (consumption) of the substrate (Agarry *et al*., 2010). The phenol utilization rate was 9.06-19.79, 16.82-25.96, 18.06-24.05, and 13.65-20.18 mg h^{-1}, respectively, at an initial phenol concentration of 200, 400, 600 and 800 mgL^{-1}, respectively (Fig. 3). It is clear that the rate of phenol utilization decreased as the initial concentrations of phenol increased. A similar observation was reported in *Acinetobacter* species (Adav *et al*., 2007; Viggor *et al*., 2020) and a mixed culture of *P. aeruginosa* and *P. fluorescence* (Agarry *et al*., 2008). In bacterial growth, the lag phase was a period of adaptation required for bacterial cells to begin to exploit new environmental conditions. Fig. 3 indicates that phenol degradation and biomass growth were initiated with a lag phase. The lag phase corresponding to 200 mgL^{-1} phenol was 7 h and increased to 18 h and 20 h at 600 and 800 mgL^{-1} of phenol concentration. The lag phase was observed to increase with an increase in phenol concentration. The result agrees with reports of Bakhshi *et al*. (2011) in *P. putida*, and Dey and Mukherjee (2010) in microbial sludge batch culture. Conversely, there are claims that cell growth occurred in the presence of phenol without any lag phase (Lin and Cheng,

2020). The lack of lag step is a positive sign suggesting that cells are adapted to phenol and are capable of rapidly degrading phenol. The total degradation time at the initial phenol concentrations of 200, 400, 600 and 800 mgL^{-1} was 15 h, 21 h, 34 h, and 51 h and the corresponding biomass growth rate during this period was 4.57-20.64, 5.51-22.77, 11.56-40.52 and 14.73-46.70 mg h^{-1} (Fig. 3). The experimentally observed specific growth rate (μ) increases with an increase in the phenol concentration until the maximum specific growth rate of 0.26 h^{-1} was observed at 400 mgL^{-1} of initial phenol concentration (Fig. 4). Similar results were reported in several species, such as *Psuedomonas putida* (Bakhi *et al., 2011*), immobilized *Pseudomonas* sp.NBM 11 (Mohanty and Jena, 2015) and *Pseudomonas* and *Bacillus* sp. (Hasan and Jabeen, 2015). This decline in specific growth rate may occur due to cell damage, protein denaturation, and disruption of membrane integrity at higher phenol concentrations and may result in reactive oxygen species (ROS) accumulation, damaging the mitochondrial and the endoplasmic reticulum (Wang *et al*., 2020). However, bacterial survival at higher phenol concentrations as the only energy source represents a synthesis of phenol degrading enzymes (Chakraborty *et al*., 2015). Microbial phenol degradation under aerobic conditions can occur via either ortho or meta pathways (Sridevi *et al*., 2012). *Acinetobacter* sp. AQ5NOL1 phenol degradation is reported via the meta-pathway (Ahmad *et al*., 2017). Analysis of transcriptome in *Acinetobacter* sp. DW-1 has shown that phenol biodegradation occurred mainly via an ortho pathway through induction of phenol hydroxylase and catechol-1,2- dioxygenase (Gu *et al*., 2017).

Experimental data on the specific growth rate at each phenol concentration was used to fit classical kinetic models using the MATLAB 7.0 curve fitting tool. The lower value of RMSE is indicative of a better fit. The results of the kinetic analysis for specific growth rate versus substrate concentration indicated that Teisser, Haldane, and Webb models have a close fit to the experimental values (Table 1 & Fig.4a). However,the specific cell growth rate does not fit well with Aiba model, and (b) Monod, and Webb models (Table 1 and Fig.4b). The results of the present study that the Haldane model is the best, have also been corroborated by observations of the other investigators (Sathya *et al*., 2015; Peng *et al*., 2018). In the present study, as predicted by the Haldane model, the kinetic growth parameters of the phenol degrading strain, *A, baumannii* W29 are maximum specific growth rate (μm) 0.96 h-^{1,} saturation constant (K$_s$) 468.6 mgL^{-1} and inhibition constant (K$_i$) 239.5 mgL^{-1}. The value of the maximum specific growth rate (μm 0.96 h^{-1}) obtained from this study was close to that reported by other investigators (Szczyrba *et al*., 2016; Nandi *et al*., 2020). The high value of the specific
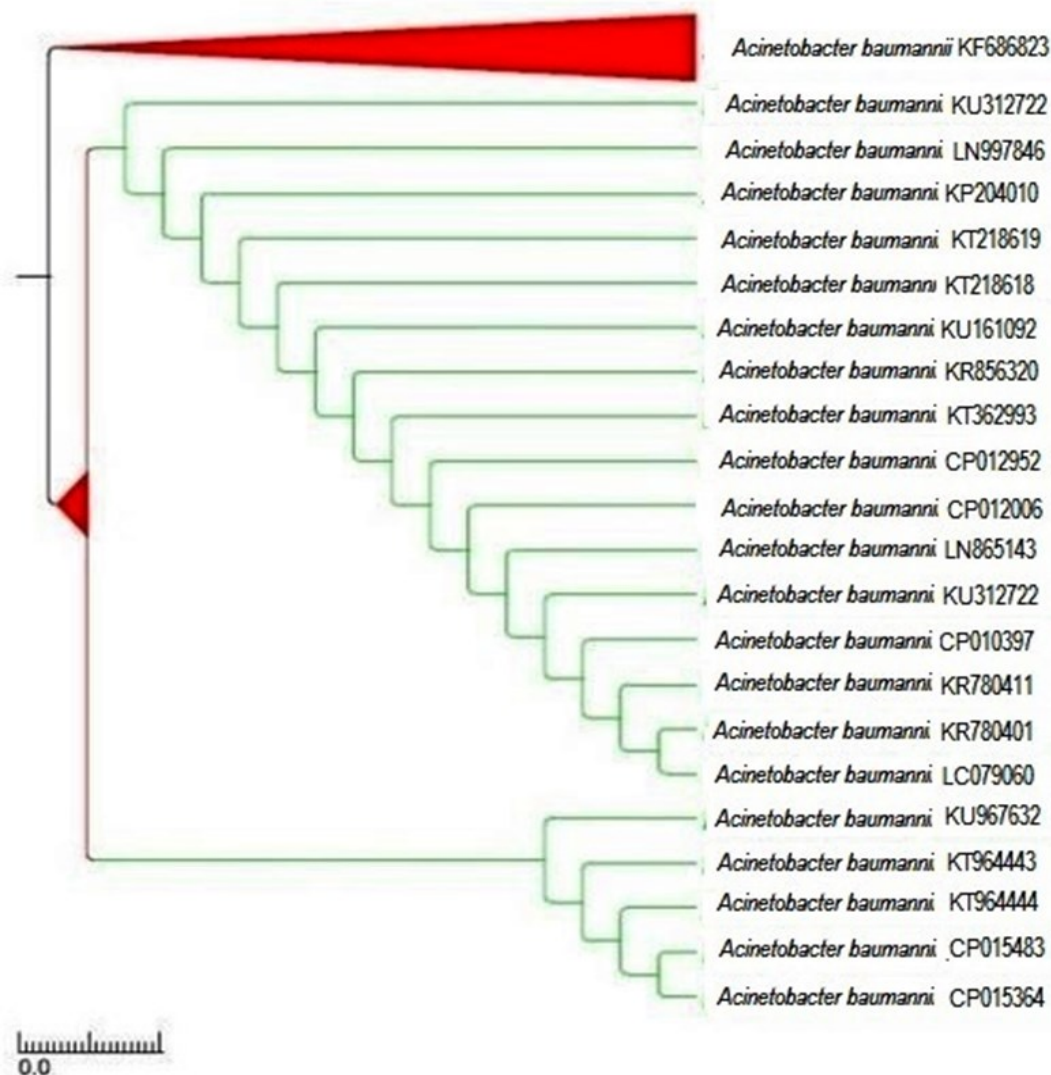
Fig. 2. *Phylogenetic tree of Acinetobacter baumanni W29*

growth rate indicated that the strain degraded the substrate more rapidly. The study showed high substrate-affinity constant (K_s 468.6 mgL^{-1}) indicating that the strain only responds to high phenol concentrations, but has low affinity for the phenol (Table 1) which is comparable to the high K_s value reported in *P. putida* (MTCC 1194) by Banerjee *et al*. (2001) and in activated sludge culture. A larger K_i value indicates that the culture is less sensitive to substrate inhibition (Onysko *et al*., 2000). High K_i showed that the strain was resistant to substrate inhibition and has shown lower phenol toxicity (Table 1).

The K_i value (239.5 mgL^{-1}) obtained from this study is comparable to that reported for the aerobic cultures, such as in *P. putida* (ATCC 49451) (K_i 284.3 mgL^{-1}) (Wang and Loh,1999) and *P. putida* BCRC 14365, K_i 255.0 mgL^{-1}) (Lin and Cheng, 2020). The difference in kinetic parameters may be explained due to different sources of inoculum and media and the physical variables during the culture. Meta-analysis of High tolerance

to phenol exhibited by various microbes, such as *Acinetobacter* calcoaceticus PA which can tolerate 1700 mg·L^{-1} phenolic wastewater (Liu *et al*., 2016) ; *Acinetobacter* EMY strain with ability to treat 2.1g/L of phenol over 86 (Kuc *et al.* 2022);and other potential phenol degrader, such as *Rhodococcus aetherivorans (*Nogina *et al*., 2020); *Sulfobacillus acidophilus* TPY (Zhou *et al*., 2016) and *Pseudomonas putida (*Mohanty and Jena, 2017) and immobilized *Acinetobacter for phenol degradation* (Abd El- Haleem et al. 2003) etc. (Table 2).

The yield coefficient is the biomass formed by the unit mass of the chemical consumed during degradation. The yield coefficient was 0.70 (mg cell mg^{-1} phenol) (Fig.5), which is in close agreement with the reports of Adav *et al*. (2007) on *Acinetobacter* species. However, the yield coefficient of phenol 0.244 (mg cell mg^{-1} phenol) is reported in *Pseudomonas putida* (Acharya *et al*., 2019). Similarly, a linear relationship between the rate of degradation of the substrate and the rate of cell growthis is reported by Wang *et al*. (2008).

In the present study, *A. baumannii* W29, isolated from wastewater, can utilize phenol as the sole source of carbon and energy. The screening criteria were based on phenol enrichment method and acclimatization, and efficiency of phenol utilization strain W29 with efficient phenol degradation capacity. Further, it is characterized by 16S rDNA sequencing and sequence alignment. The results of the study showed that the specific growth rate increased with an increase in phenol concentrations in the range of 100-400 mgL^{-1} with a maximum specific growth rate at 400 mgL^{-1} of phenol.

Experimental data specific growth rate at 100–800 mg L^{-1} phenol was fitted to conventional growth models. The results showed that the Haldane equation was the best model for predicting experimental data. The high

value of K_s indicates that the strain responds well to a high phenol concentration. The high value of K_i and the large μ_{max} value indicates that the strain can degrade a high phenol concentration. The future scope of the study was to use the strain in the mixed consortium of bacteria that may further reduce the substrate inhibition effect of phenol. The experimental method and the kinetic model developed in this study may be further used in the immobilized cell/bioreactor for large-scale removal of phenol from wastewater in different industries.

Conclusion

The present study showed that isolated strain *A. bau-*

Table 1. Kinetic parameters of the specific growth rate of culture growth by *Acinetobacter baumannii* W29

Model	Equation	μ_m	Ks	Ki	R2	RMSE
Teisser	$\mu = um*(exp(-S/Ki) - exp(-S/Ks))$	0.356	132.9	1372	0.972	0.01444
Haldane	$\mu = (um*S)/(Ks+S+S^2/Ki)$	0.963	468.6	239.5	0.9755	0.0135
Aiba	$\mu = (um*S)* exp(-S/Ki)/(Ks+S)$	0.596	226.7	936.6	0.9669	0.01569
Monod	$\mu = um*S/(Ks+S)$	0.228	20.64		0.8191	0.03429
Webb	$\mu = (um*S)*(1+S/Ki)/(Ks+S+S^2/Ki)$	0.216	18.43	21.2	0.8078	0.03779
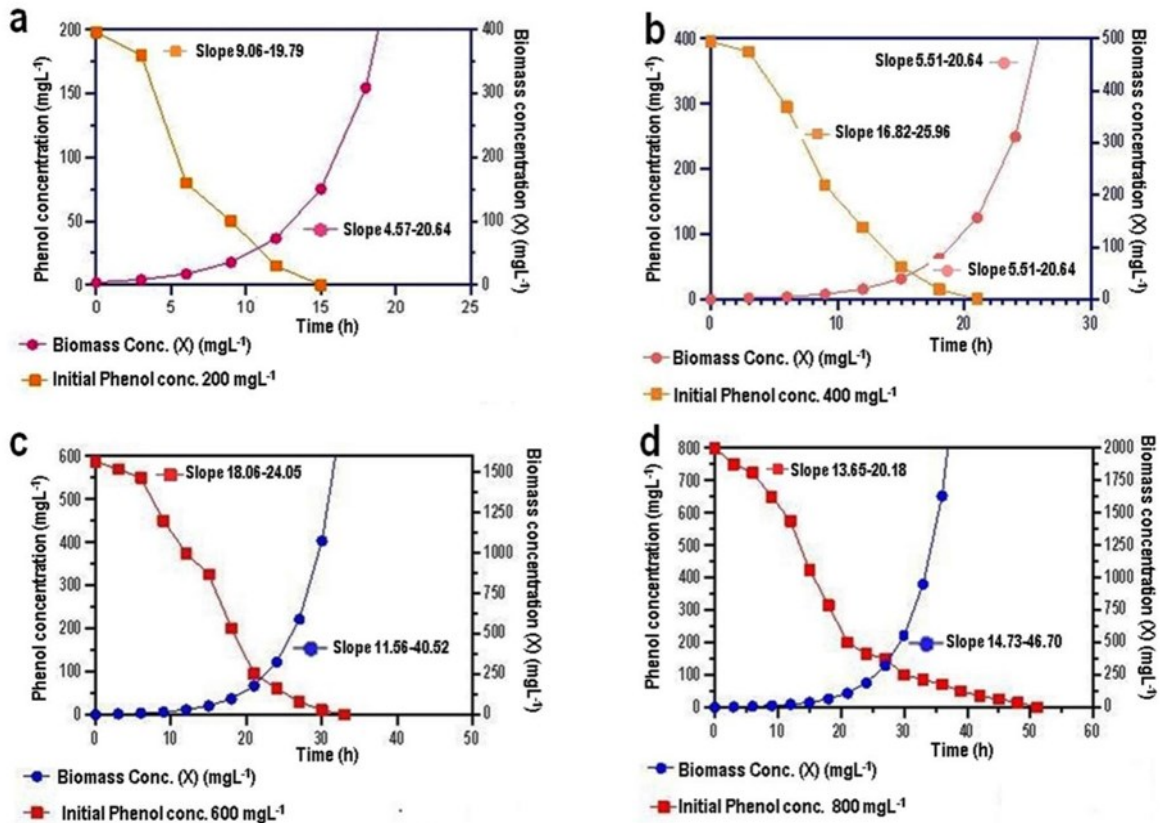
Fig. 3. *Biomass growth and utilization of phenol by Acinetobacter baumannii W29*

Table 2. High tolerance to phenol exhibited by various microbes in various studies

Strain	Source	Phenol tolerance level	Reference
Acinetobacter sp.	Wastewater treatment plant	500 mg·L−1	Abd El-Haleem *et al.* (2003)
Acinetobacter Calcoaceticus PA *Rhodococcus aetherivorans*	Phenolic wastewater lubricant-contaminated soil environment	1700 mg·L−1 1750 - 2000 mg L−1	Liu *et al.* (2016) Nogina *et al.* 2020
Sulfobacillus acidophilus TPY,	hydrothermal vent in the Pacific Ocean	1300 mg/L	Zhou *et al.* (2016)
Pseudomonas putida	NCIM 2650	1000 mgL-1	Mohanty and Jena (2017)
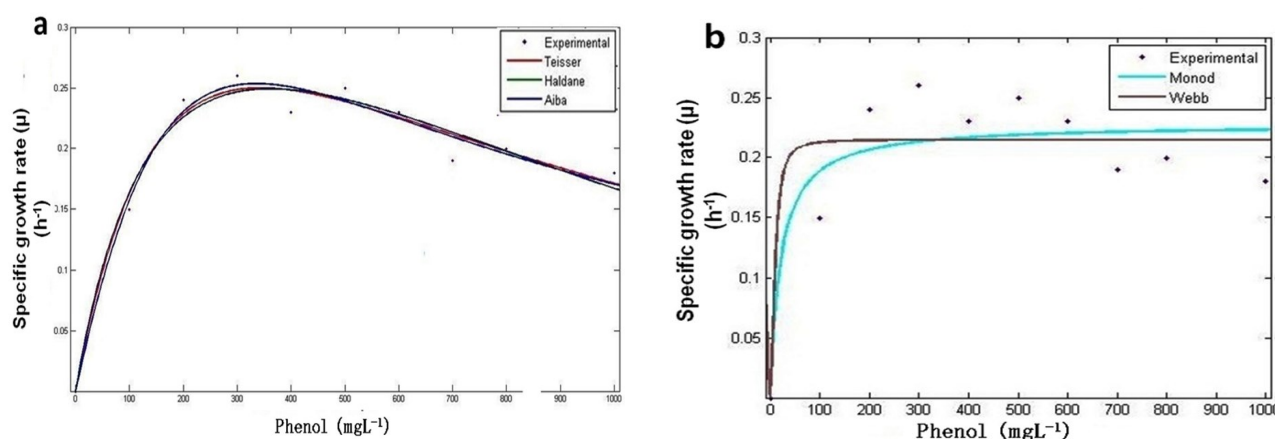
Fig. 4. *Specific cell growth rate of Acinetobacter baumannii W29 in presence of phenol. (a) Teisser,Haldane and Aiba model, and (b) Monod, and Webb models*
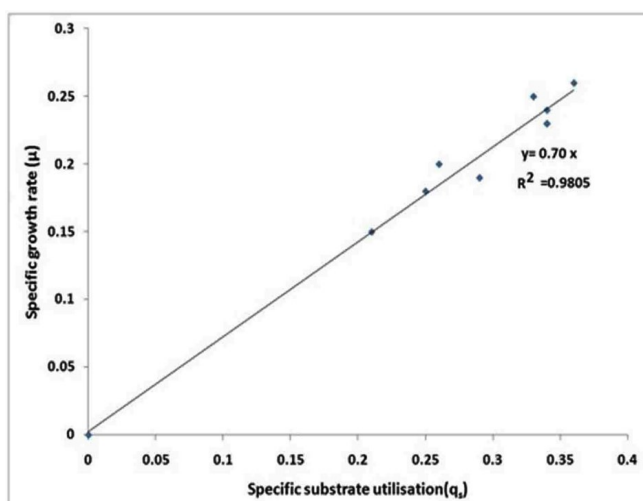
Fig. 5. *Yield coefficient Acinetobacter bauminnii W29 growing in phenol containing media*

mannii W29 has a high capacity for phenol degradation. The strain uses phenol as source carbon and energy for its growth. The higher values of K_s, K_i, and μ_m indicated that the strain could respond well to high phenol concentrations. In the study, Haldane, Teisser, and Aiba were the most suitable kinetic models for the biodegradation of phenol. Because of above study, it is concluded that *A. baumannii* W29 can be successfully used to treat wastewater containing phenol.

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Conflict of interest
The authors declare that they have no conflict of interest.

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