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Evaluation of Cr(VI) Reducing Capability of *Bacillus licheniformis* DAS1 Using a Multifactor Experimental Approach

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ABSTRACT

The current study is about detoxifying soil and water contaminated with toxic Cr(VI). To ensure that DAS1 could develop as well as possible, the pH was changed between 4 and 10. DAS1 showed its highest growth at pH 8, and at the same pH, it had an 85% potential to remediate by converting Cr(VI) to Cr(III). Immobilized bacteria increased the reduction of Cr(VI) to Cr(III) from the culture medium to 90.4%. The impact of glucose concentrations between 0.5 and 2.5 $g.L^{-1}$ was examined. The greatest development was seen at pH 8 and 2 $g.L^{-1}$ glucose concentration. The remediation potential was improved by up to 96% when the growing medium contained 200 $mg.L^{-1}$ Cr(VI). The value of k_s (0.434 $g.L^{-1}$) demonstrated the substrate's affinity for bacteria in accordance with the Monod equation, while μ max (0.090 h) demonstrated that DAS1 required 11.11 h for maximal growth. The multifactor experimental design was used to analyze mixed cultures of DAS1 and DAS2 in a 1:1 ratio, and it was determined that the X3Y2Z1 experiment design was best for completely removing Cr(VI) from the growing medium. By making pores using Na_2EDTA, it was determined that the cell membrane's impermeability did not cause Cr(VI) resistance in DAS1. The delayed lag phase indicated that the enzyme activity was inductive rather than constitutive.

INTRODUCTION

India has a population of about 1.4 billion and is growing at a 1.6% annual rate. Every person utilizes leather shoes, leather clothing, and leather things from tanneries. India's tanning industry significantly pollutes the environment with Chromium. In India (at 25.6197° N, 85.1660°E), there are 1083 tanneries, according to the Central Leather Research Institute (1987). Among the states with the highest concentration of tanneries are Tamil Nadu (577 units), West Bengal (233 units), and Uttar Pradesh (147 units). These three states are home to 88% of the nation's tanneries. Most of these industries lack effluent treatment facilities, resulting in an average daily outflow of 50-60 million liters of untreated wastewater.

As per a WHO report from 2001, 90% of the 8000 tannery workers in Hazaribagh, India (25.6170° N, 85.1660° E) died before the age of 50 due to gastrointestinal, dermatological and other disorders like cancer. Chromium is the main contaminant in tannery wastewater. Both mobile and chelated forms of chromium occur. With the tannery sector, India's (25.6197° N, 85.1660° E) paper and pulp, electroplating, and several small-scale industries located in Bihar also contribute to Chromium contamination. About a lakh weavers in Eastern India (Bihar) make a living by producing and dealing with fabric and clothing (25.6173° N, 85.1660° E). Numerous small-scale companies in the Patna metropolitan area of Bihar discharge untreated wastewater into the sewer system, contributing to chromium pollution. Every aquifer and other aquatic system is linked together in some way. In addition to the anthropogenic activities, spills, and dumping of chromium wastes, geological processes also aid in introducing chromium and its compounds into soils (Ayele & Godeto 2021). If present below 100 ppm, chromium is functional in the metabolism of lipids, carbohydrates, amino acids, and nucleic acid production (Eaton et al. 2005). Since chromium is more soluble and has a persistent oxidation property in the trivalent (Cr^{3+}) form, which is predominant in biological systems, it exhibits lower biotoxicity (Elahi et al. 2022, Ray 2016).

Those exposed to Cr show signs including necrosis, eardrum perforation, and skin and ear ulcers. Cr pollution is causing havoc in the ecosystem by negatively affecting the abundance and balance of microbial populations in

different ecosystem components, such as soil and water. One of the most significant ecological concerns is the effective removal of heavy metals from industrial waste, particularly from aqueous waste. Several old and typical procedures for removing heavy metals, including solvent extraction, oxidation-reduction processes, and adsorption, are generally expensive and inefficient, especially when the concentration of heavy metals reaches 1 $mg.L^{-1}$ (Elahi et al. 2022).

In water containing industrial effluent, bacteria live. These bacteria have evolved survival mechanisms to deal with the toxicity of heavy metals through processes such as metal uptake, methylation, adsorption, oxidation, and reduction. For the detoxification and eradication of Cr(VI) contaminants, bioremediation technology is a straightforward, affordable, and environmentally acceptable approach that may be used in a variety of experimental settings.

The most notable bioremediation methods include biosorption, bioaccumulation, biotransformation, and bioreduction. Out of everything said above, this study investigates the role of bio-reduction of Cr(VI) to Cr(III). Using local microbiota to remove heavy metals from contaminated environments and restore the polluted area without the addition of chemical reagents, bio-reduction, or the conversion of Cr^{6+} to Cr^{3+} is a potential detoxification technique (Tripti et al. 2014, Elahi et al. 2022).

In addition to microorganisms, plants like *Typha latifolia* L. and *Phragmites australis* are reported to mitigate metalloids like selenium by biotransformation (Elahi et al. 2022, Sayantan & Shardendu 2013), and certain annual plants like Raphanus sativus are reported to mitigate Cr(VI). The toxicity of Cr(VI) was reported dose-dependent. There was a gradual reduction in Cr(VI) toxicity on phosphate amendment at each increasing concentration of P amendment (Sayantan & Shardendu 2013). The enrichment of PO43- above 1 mM did, however, cause a growth reduction in *Bacillus licheniformis* DAS1, demonstrating the toxicity of phosphate (Akcil et al. 2015, Tripti & Shardendu 2016).

The current experiment uses *Bacillus licheniformis* DAS1 to bioreduce Cr(VI) at various pH and Cr concentrations. Immobilized bacterial cells on calcium alginate beads were also used to bioreduction Cr(VI) to Cr(III). Glucose was employed as a co-metabolic substrate to calculate the maximal specific growth rate (max) and ks (Shivangi et al. 2002).

The notion of microbial growth kinetics has been dominated by an empirical model first proposed by Monod (1942). The Monod model was the first to suggest the existence of a substrate that restricts growth.

$$\mu = \mu_{max}[S]/k_s+[S]$$

Specific growth rate (μ) is independent of substrate concentration if there is excess substrate present. From the hyperbolic curve generated by Monod's equation, it is impossible to accurately calculate what ks and μ max should be. Use the Line Weaver Burk plot to linearize (Double Reciprocal Plot). The formula for Monod is

$$1/\mu = K_S/\mu_{max}\,1/[S] + 1/\mu_{max}$$

Also, an orthogonal experiment design was used in a mixed culture of DAS1 and DAS2 to maximize the reduction of Cr(VI) to Cr(III) (1:1) (Shimei et al. 2012). The mechanism underlying the resistance to Cr of DAS1 was also investigated using a permeability experiment (Batool et al. 2012).

Objective of the Current Work

The objective of current research work is to evaluate the reduction of Cr(VI) to Cr(III) by optimizing the pH temp and inoculum concentration of bacteria *Bacillus licheniformis* DAS with a multifactor approach.

MATERIALS AND METHODS

Determination of MTC (Maximum Tolerable Concentration) of DAS1 for Cr(VI)

A solid nutritional agar plate was streaked with the isolated *Bacillus licheniformis* DAS1 after it had been treated with a range of Cr(VI) concentrations (50-1100 $mg.L^{-1}$). The maximum tolerated concentration of a pollutant was determined to be the level at which bacterial growth could be seen after 5 days of incubation at 37°C (Zhao et al. 2016).

Effect of pH and Cr(VI) Concentration on Specific Growth Rate(μ) and Cr(VI) Reduction

In LB broth, the effects of various pH values (ranging from 4 to 10) and the initial Cr(VI) concentration (200 to 1000 $mg.L^{-1}$) were studied (Tripti & Shardendu 2016, Wani et al. 2019), and the specific growth rate (μ, h^{-1}) was estimated using the slope of the graph (Upadhyay et al. 2017, Villegas et al. 2013).

In OD/OD_o

where OD=optical density at the given time and OD_o=optical density at zeroeth h

Effect of Glucose as Co-metabolic Substrate

Co-metabolic tests were carried out using a binary substrate system that contained bacteria, Cr(VI) at 200 $mg.L^{-1}$, and glucose in the range of 0.5-2.5 $g.L^{-1}$ to improve the specific

growth rate and reduction capacity of DAS1 (Upadhyay & Sinha 2021). The Monod kinetic parameters kS and μmax were tuned using a line weaver Burk plot.

$$\mu = \mu_{max}[S]/k_s+[S] \text{(Monod equation)}$$

$$1/\mu = K_S/\mu_{max} 1/[S] + 1/\mu_{max} \text{ (Line Weaver Burk Plot)}$$

Chromium(VI) Reduction

The concentration of hexavalent Cr becoming less hazardous was estimated using the DPC method by DAS1. A 24-h-old culture was inoculated into 50 ml of LB broth with 200–1000 $mg.L^{-1}$ of Cr VI and a pH range of 4-10. The samples were obtained by centrifuging for ten minutes at 10,000 rpm. By measuring the absorbance of the Cr(VI)-DPC complex at 540 nm with a spectrophotometer and plotting the results in the standard curve at various time intervals, it was possible to calculate the remaining Cr(VI) concentration.

The percentage reduction of Cr(VI) was calculated by using the formula (Upadhyay et al. 2017)

$$\text{Cr(VI) reduction } \% = C_0\text{-}C_f/C_0*100$$

Where C_0 = Initial Cr(VI) concentration, C_f = Final Cr(VI) concentration.

Determination of the Ratio of Two Strains

To evaluate the effectiveness of Cr(VI) bioreduction, a mixture of the two strains, DAS1 and DAS2, in various ratios containing 1:1, 1:2, and 2:1, were used. Then, the ratio with the highest Cr(VI) reduction efficiency was chosen.

Orthogonal Experiment Design

Compared to traditional design, orthogonal experiment design excels in optimizing the growth-related parameters, particularly temperature, pH, and inoculation amount (Table 1), and was created to find the ideal circumstances for mixed strains to remove Cr(VI).

Effect of Immobilization of *Bacillus licheniformis* DAS1

During 24 h at 37°C, DAS1 was grown aerobically in LB broth. A 3 percent sodium alginate solution was made and autoclaved. To ensure homogeneous cell and alginate solution mixing, the bacterial culture and alginate were combined in a 1:4 ratio and shaken for 2 h. With constant shaking with a 10 mL disposable syringe, the mixture was poured dropwise into 50 mL of $CaCl_2$ solution in a beaker to create beads. For hardening, the beads were left overnight. The 3-5 mm-diameter beads were repeatedly cleaned with sterile distilled water after 24 h. Beads were kept at 4°C and were done under aseptic conditions. 50 mL LB broth with 200 $mg.L^{-1}$ Cr(VI) and 5g beads were added.

Table 1: Factorial Design for Orthogonal Experiment.

Level	Temperature	pH	Inoculation amount
1	25	7.0	5%
2	30	8.0	10%
3	35	9.0	15%

Cell Permeability Assay and Induction Behavior

In the same buffer, cells were resuspended after being harvested at the exponential phase and once at room temperature with 0.12 Tris-Cl, pH 8.0. The suspension was incubated with 2*10-4M Na_2EDTA for two minutes at 37°C before the treatment was finished with the addition of ten volumes of growth media. Controls were diluted in the same way and added without Na_2EDTA.

Instruments Used

Ultraviolet-visible (UV-Vis) Spectrophotometer is used to quantify the reduction of Cr(VI) to Cr(III) in the spiked growth medium.

RESULTS AND DISCUSSION

Growth Profile of DAS1 in the Presence of Cr(VI) Stress

In this investigation, *Bacillus licheniformis* DAS1 was chosen after being isolated from the rhizosphere of *A. viridis*. It was allowed to grow in LB broth that had been changed to include Cr(VI) at varying concentrations, such as 200 $mg.L^{-1}$, 400 $mg.L^{-1}$, 600 $mg.L^{-1}$, 800 $mg.L^{-1}$, and 1000 $mg.L^{-1}$ (Fig.1). The harmful effect of chromium was demonstrated by comparing the growth of cells cultured in Cr(VI) free media and Cr(VI) containing media. *Bacillus licheniformis* DAS1 grown in control (without chromium) was observed to spend the first two hs in the lag phase by remaining metabolically active and increasing only the size of cells, not the number of cells, before entering an exponential phase that lasted for nearly 26 h and saw the population growth in a logarithmic manner.

The toxic impact of the metal on DAS1 may have caused the lag phase in Cr(VI) at 200 and 400 $mg.L^{-1}$ to be significantly extended to 3 hrs.

After that, an exponential phase lasting approximately 25 h began. The specific growth rate (μ, h^{-1}) of DAS1 and Cr(VI) concentration was shown to be negatively correlated (Table 2), meaning that as Cr(VI) concentration climbed to 600, 800, and 1000 $mg.L^{-1}$, the lag phase also increased to 4 h, shortening the exponential phase to 22 h. With 1000 $mg.L^{-1}$, DAS1 went through a 20 h exponential phase before entering the stationary period.

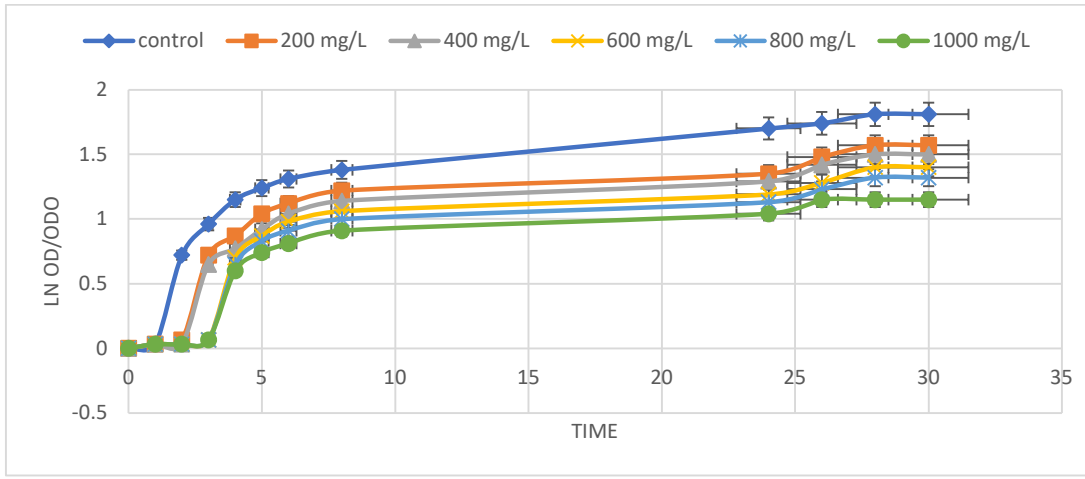

Fig. 1: Variation in growth of *Bacillus licheniformis* DAS1 at different Cr^{6+} conc at neutral pH.
(All values are mean of three replicates, and standard errors (SE) are presented as error bars ($^{+}_{-}$)

The specific growth rate (μ, h^{-1}) of DAS1 calculated and presented in Table 1 vividly portrays the negative correlation between the Cr(VI) concentration and growth of DAS1.

Effect of pH on the Growth of *Bacillus licheniformis* DAS1

Bacillus licheniformis DAS1's growth and development are influenced by pH. The pH limit of a cell's structural integrity and the interference of pH with cell metabolism are represented by the specific growth rate of these organisms changing with pH as a bell-shaped curve (Fig. 2 to Fig. 7 and Table 2). The highest growth was seen at pH 8 (Fig. 5 and Table 2), and it decreased as pH became more acidic and basic. The pH needed for bacteria to thrive was neutral, but up to a certain point, alkaline pH resulted in faster growth (pH 8). The growth decreased when pH 9 & pH 10 increased (Fig. 6 & Fig. 7).

pH Modulates Toxicity of Cr(VI)

Bacillus licheniformis DAS1 may reduce Cr(VI) to chromium(III) using the chrome reductase enzyme. Each enzyme's functionality is influenced by pH and temperature

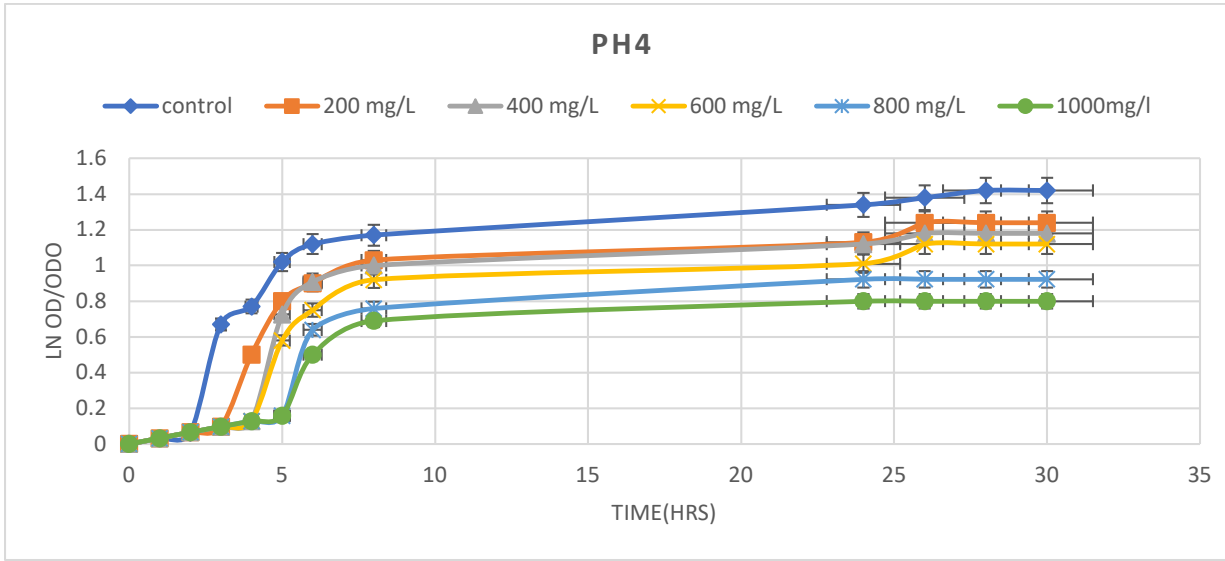

Fig. 2: *Bacillus licheniformis* DAS1 growth variation at pH 4 under different Cr^{6+} conc.
(All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±).

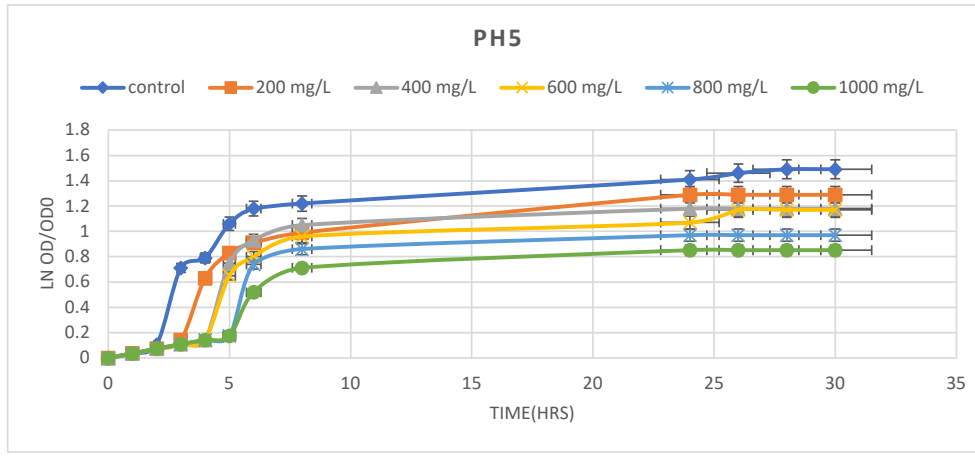

Fig. 3: *Bacillus licheniformis* DAS1 growth variation at pH 5 under different Cr^{6+} conc.
(All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±).

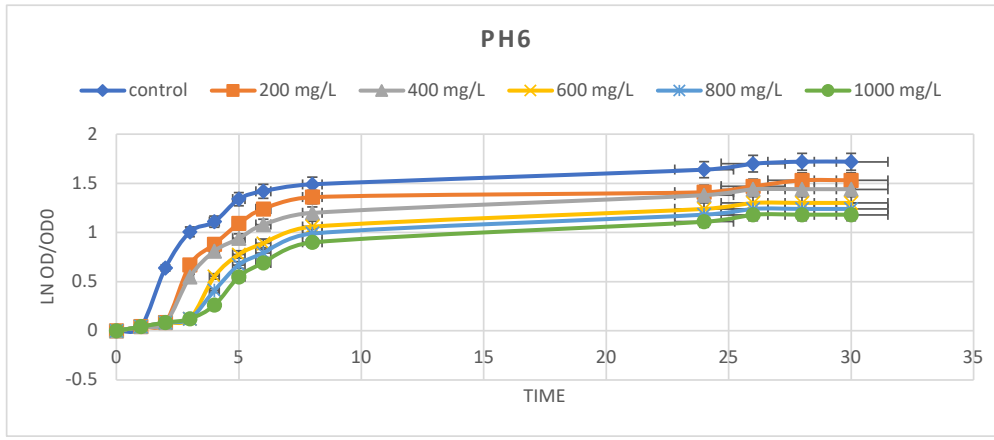

Fig. 4: *Bacillus licheniformis* DAS1 growth variation at pH 6 under different Cr^{6+} conc.
(All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±).

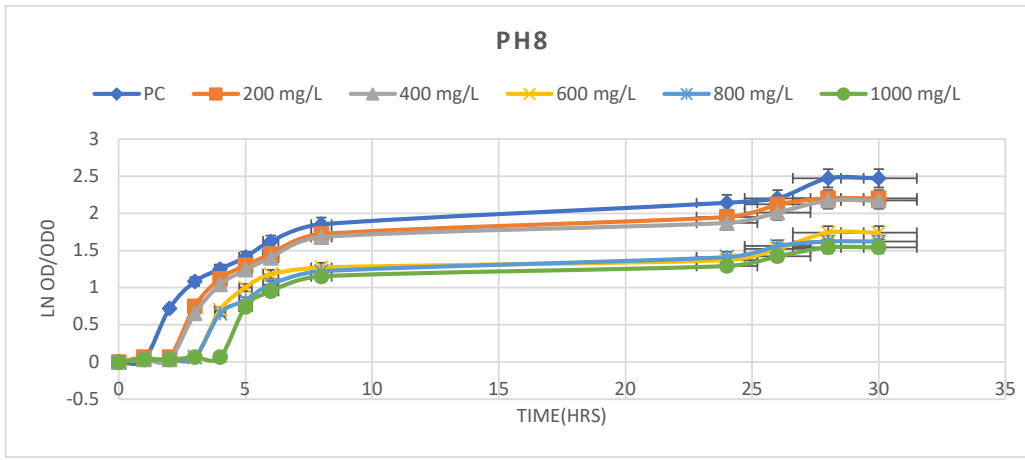

Fig. 5: The variation of *Bacillus licheniformis* DAS1 growth at pH 8 under different Cr^{6+} conc.
(All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±).

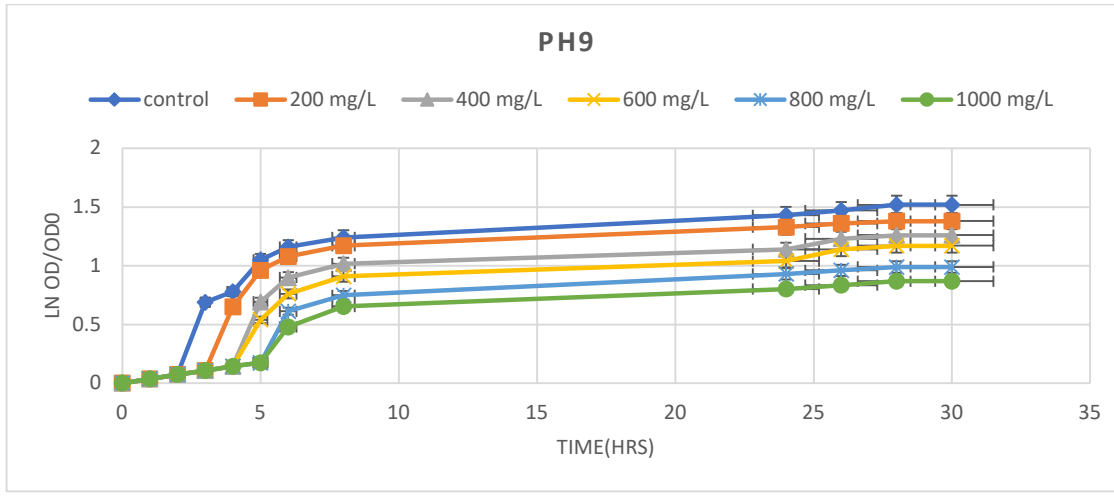

Fig. 6: *Bacillus licheniformis* DAS1 growth variation at pH 9 under different Cr^{6+} conc.
(All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±).

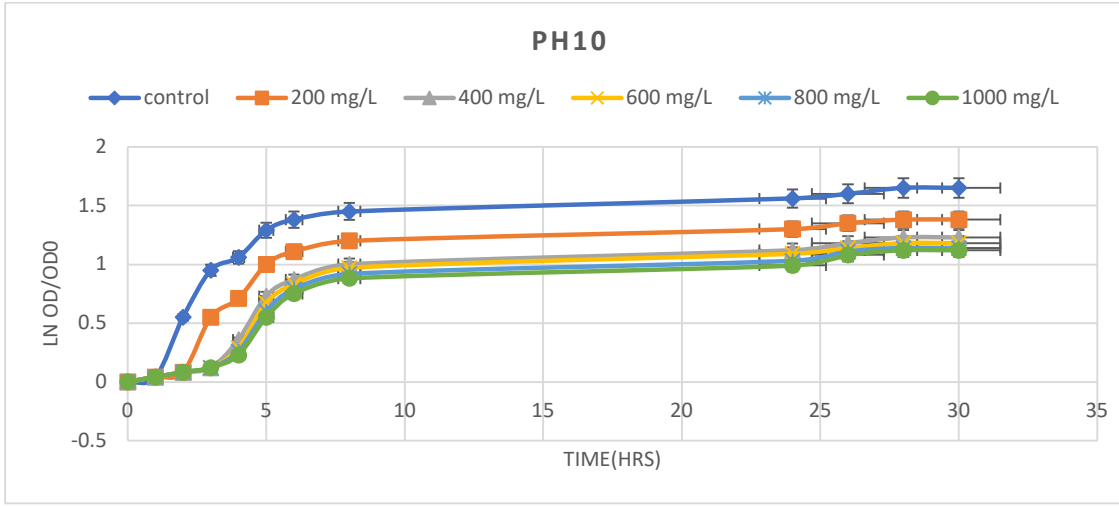

Fig. 7**:** *Bacillus licheniformis* DAS1 growth variation at pH 10 under different Cr^{6+} conc.
(All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±).

Table 2: Specific growth rate(μ): h^{-1} at different pH under different Cr^{6+} conentration. (Calculated from the slope of the graph)

Specific growth rate(μ): h^{-1} at different pH under different Cr^{6+} conentration (Calculated from the slope of the graph)							
Sample	Neutral	pH 4	pH 5	pH 6	pH 8	pH 9	pH 10
Control	0.043832	0.038003	0.039861	0.040187	0.063039	0.041229	0.038514
200 $mg.L^{-1}$	0.041538	0.037254	0.039173	0.040109	0.062125	0.040782	0.037286
400 $mg.L^{-1}$	0.040895	0.037360	0.037371	0.040060	0.061599	0.039774	0.037217
600 $mg.L^{-1}$	0.040603	0.035860	0.037217	0.039632	0.050483	0.037399	0.036237
800 $mg.L^{-1}$	0.038582	0.031244	0.032309	0.038830	0.049997	0.033061	0.035229
1000$mg.L^{-1}$	0.034338	0.026874	0.028604	0.038088	0.049529	0.028708	0.034739

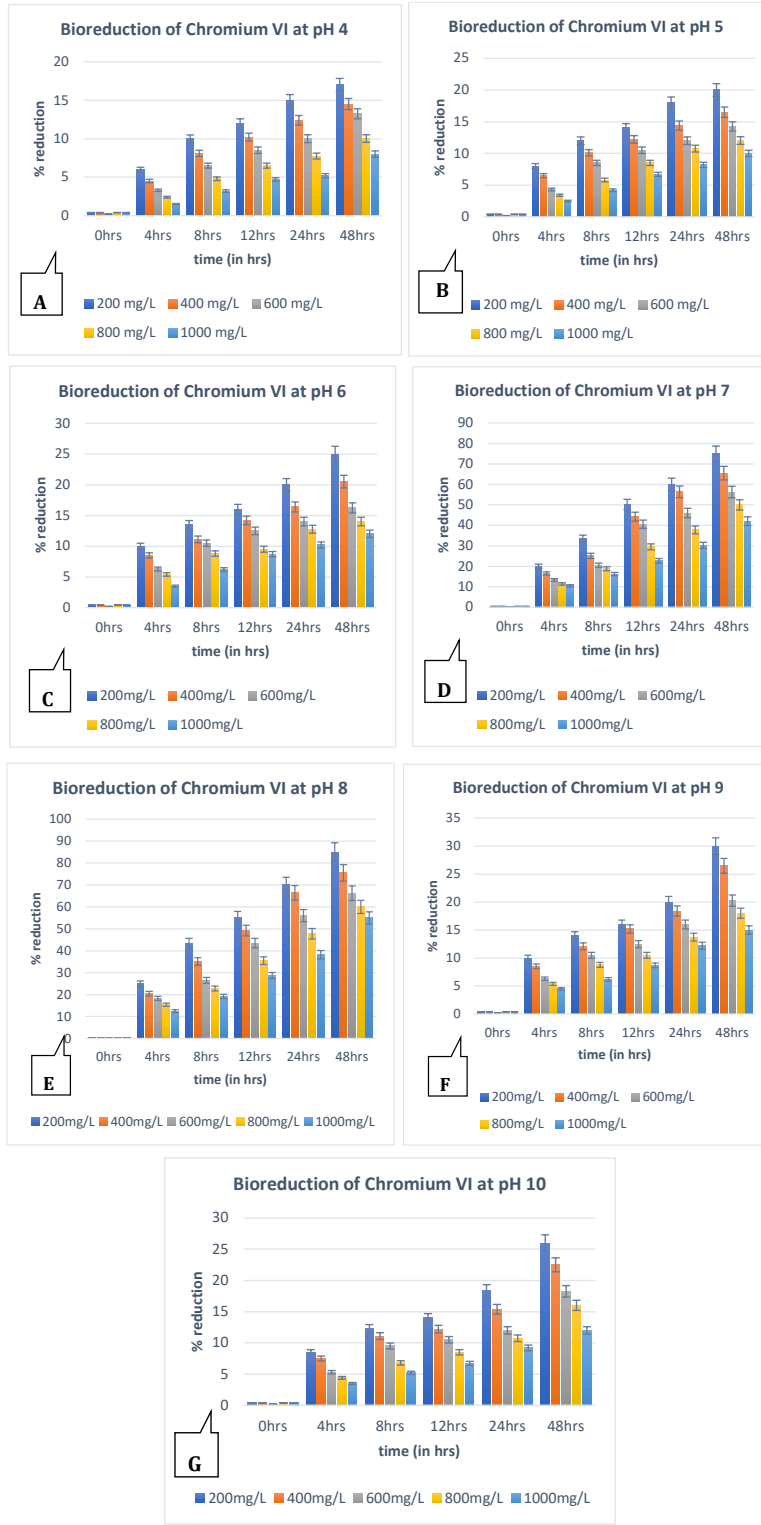

Fig. 8: Percentage reduction of Cr6+ at variable pH under different Cr $^{6+}$ Concentration.
pH4; **B**. pH5 ; **C.** pH6 ; **D**. pH7 **E**. pH8 **F.**.pH9 **G.** pH 10
(Error bars indicate the standard error of the mean reduction efficiencies from triplicate experiments with a p-value <0.05.)

Table 3: specific growth rate (μ, h^{-1}) at pH 8 with variable glucose concentration at 200 mg.L^{-1} Cr^{6+}. (Calculated from the slope of the graph).

Sl. No.	Samples	Specific growth rate(μ): h^{-1}
1.	control	0.063039
2.	0.5 g.L^{-1}	0.063039
3.	1 g.L^{-1}	0.075187
4.	1.5 g.L^{-1}	0.078524
5.	2 g.L^{-1}	0.082863
6.	2.5 g.L^{-1}	0.082863

(from Fig. 8A to Fig. 8G). The highest decrease of up to 85% was seen at 200 mg.L^{-1} of Cr^{6+} at pH 8 at roughly 48 hs (Fig. 8E), and the smallest reduction of 8.01%% was found in 1000 mg.L^{-1} of Cr(VI) at pH 4. (Fig. 8A). Nonetheless, the 48 h period was consistent for all the data.

Effect of Co-metabolic Substrate on Growth of DAS1 Reduction of Cr(VI)

Recently, cometabolism has become a potent technique for the biodegradation of refractory contaminants. Changing the substrate's carbon and energy composition may increase refractory compound degradation efficiency. The proportional relationship between a specific growth rate and a low substrate concentration is described by the Monod Equation.

$$\mu = \mu_{max}[s]/K_{s+} S$$

Three parameters are typically discovered to affect the specific growth rate of DAS1: (a) the concentration of the limiting substrate (glucose), (b) the maximal specific growth rate (max), and (c) the substrate-specific constant (Ks). The specific growth rate is independent of substrate concentration if excess substrate exists. Monod Equation yields a hyperbolic curve from which it is impossible to determine the precise values of Ks and μ_{max}. The Monod equation was linearized using a line weaver-Burk plot, and the values of Ks and max were determined to be 0.44 g.L^{-1} and 0.09 h^{-1}, respectively, using the equation below:

$$1/\mu = K_s /\mu_{max.}\ 1/s + 1/\mu_{max}$$

Apart from the mathematical meaning, k_s describes the affinity of microorganisms for a particular substrate. Fig. 9 to Fig. 12 illustrate the Effect of Co-metabolic substrate on the growth of DAS1 reduction of Cr(VI). Specific growth rates (μ, h^{-1}) at pH 8 with variable glucose concentration at 200 mg.L^{-1} Cr^{6+} are given in Table 3.

Determination of the Optimum Ratio of Mixed Culture for Maximum Cr(VI) Reduction

DAS1 and DAS2 were examined separately for their capacity for a reduction before being employed as mixed cultures in various ratios, including 1:1, 1:2, and 2:1. The ratio of 1:1 was chosen since it allowed for a maximum reduction of 97% (Fig. 13).

Orthogonal Test

An experimental design known as an "orthogonal test" assures that all stated parameters may be calculated independently. The

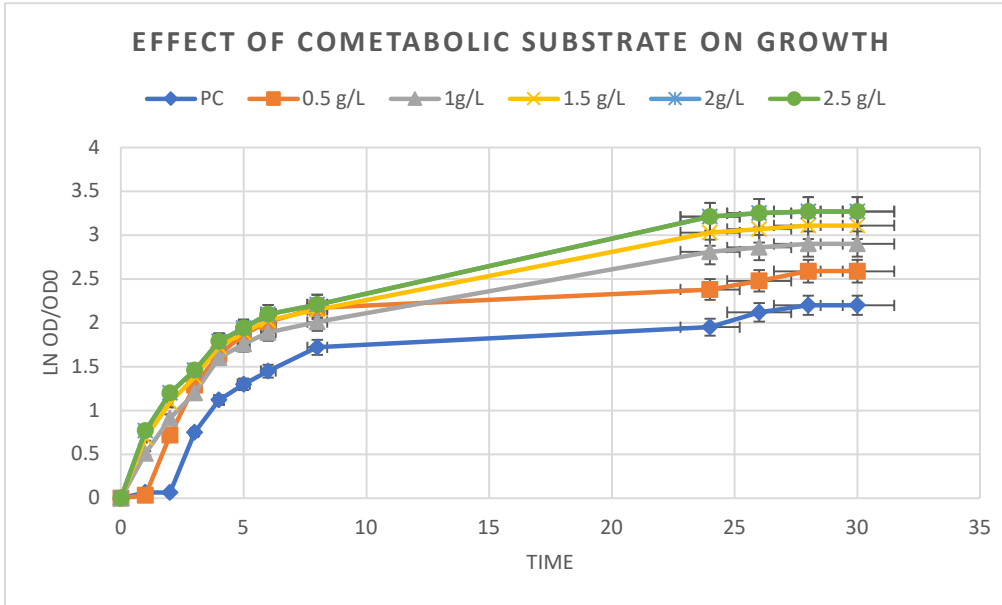

Fig. 9: *Bacillus licheniformis* DAS1 growth variation at pH 8 with variable glucose concentration at 200 mg.L^{-1} Cr^{6+}. (All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±)

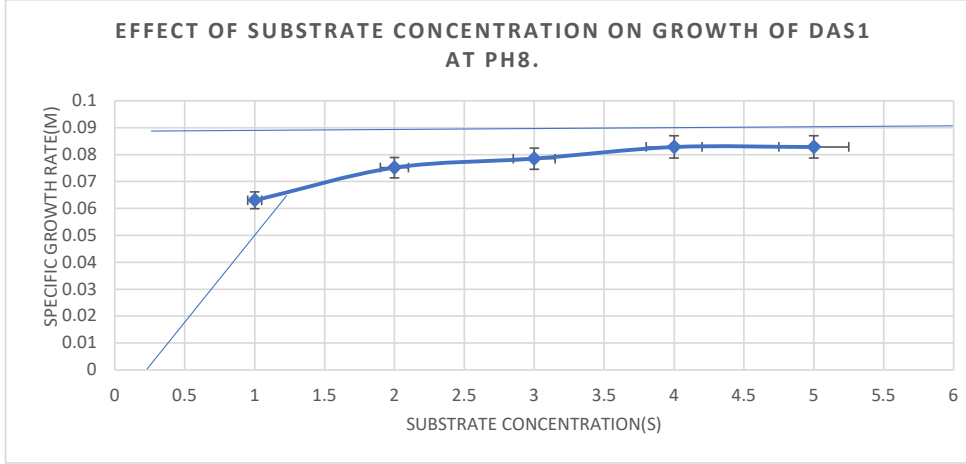

Fig. 10: Effect of substrate (glucose) conc on *Bacillus licheniformis* DAS1 growth at pH 8 measured as k_s and μ_{max} by Monod equation.
[All values are the mean of three replicates, and standard errors (SE) are presented as error bars (±)]

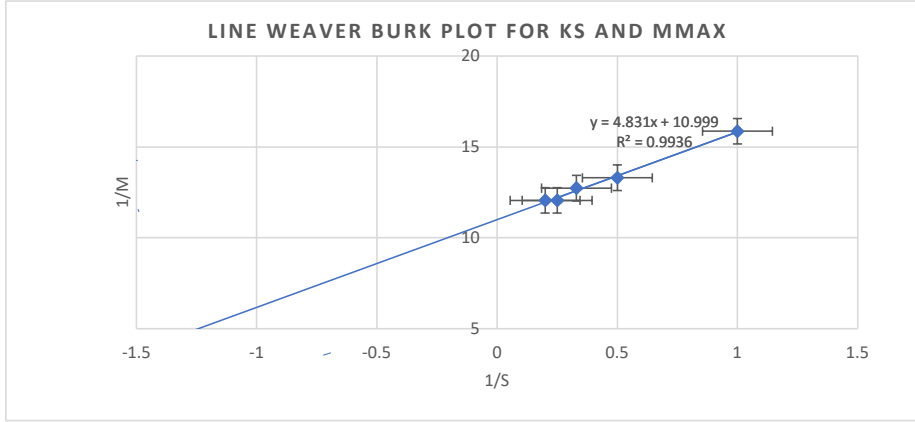

Fig. 11: Line Weaver Burk plot to Calculate k and μ_{max}.
[All values are the mean of three replicates and standard errors (SE) are presented as error bars (±)]. R^2=0.9936

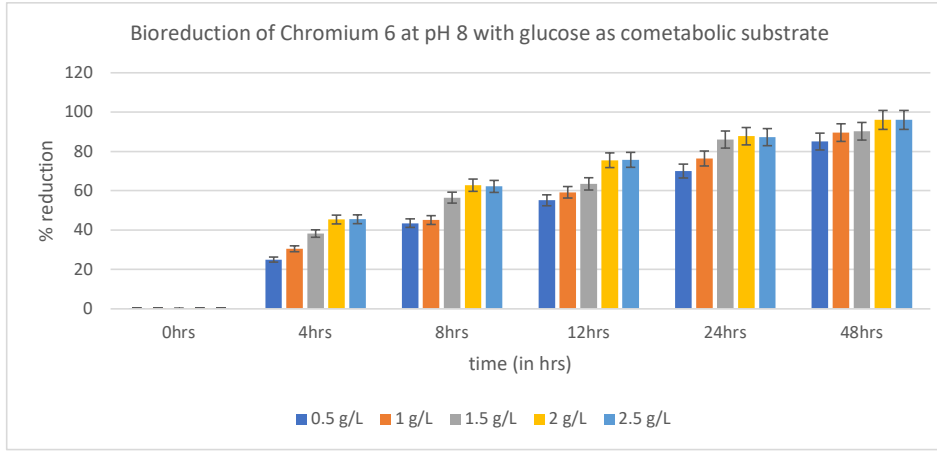

Fig. 12: percentage reduction of Cr^{6+} at pH 8 & 200 $mg.L^{-1}$ Cr^{6+} with glucose as Co-metabolic substrate.
(Error bars indicate the standard error of the mean reduction efficiencies from triplicate experiments with a p-value <0.05.)

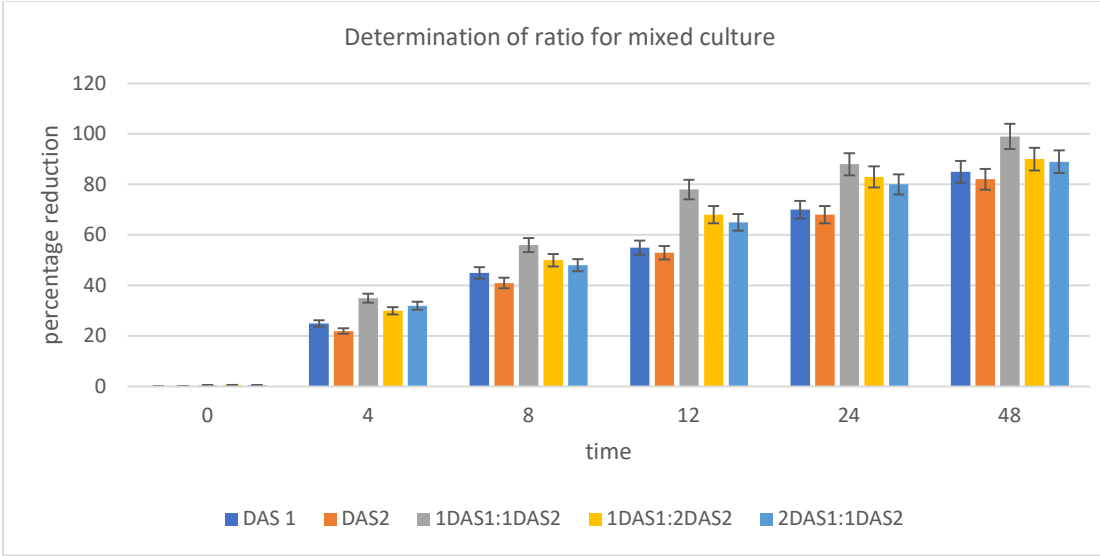

Fig. 13: Effect of mixed culture (1:1) of DAS1 and DAS2 on reduction of Cr^{6+} toxicity.
(Error bars indicate the standard error of the mean reduction efficiencies from triplicate experiments with a p-value <0.05.)

Table 4: Experimental design, X-temp (X1-25° C, X2-30° C, X3-35° C) Y-pH (Y1-7.0 ,Y2-8.0, Y3-9.0) Y-Mixed inoculum of DAS1 and DAS2 in 1:1 ratio (Z1-5%,Z2-10%,Z3-15%).

Test No.	X Temperature(°C)	Y pH	Z Inoculation Amount (%)	Combination	Cr(VI) -removal rate (%, 2d)
1	2	1	2	X2Y1Z2	65
2	1	2	2	X1Y2Z2	88
3	3	1	3	X3Y1Z3	76
4	1	1	1	X1Y1Z1	68
5	2	3	1	X2Y3Z1	56
6	3	3	2	X3Y3Z2	59
7	1	3	3	X1Y3Z3	48
8	2	2	3	X2Y2Z3	89
9	3	2	1	X3Y2Z1	99

mixed inoculum's optimal temperature, pH, and dose were determined through an orthogonal experiment design (33). The combination X3Y2Z1 demonstrated maximum efficiency, reducing 99% of 200 $mg.L^{-1}$ Cr(VI) in under 48 h. Therefore, the ideal growing conditions for DAS1 and Cr(VI) reduction were pH 8, 35°C, and a 5% inoculum concentration (Table 4).

Effect of Bacterial Immobilization on Cr(VI) Reduction

By immobilizing DAS1 in calcium alginate beads with a diameter of 3 to 5 mm, the Cr(VI) reduction capacities of mobile and immobile bacteria were assessed. It was discovered that after 48 hrs of incubation, immobilized bacterial cells could reduce 90.4% of Cr(VI), whereas mobile cells could only reduce up to 85% of Cr(VI) (Table 5). The findings suggest that encapsulated cells have more reduction potential than mobile cells, which may be explained by the fact that stationary cells devote all their energy to reducing CR(VI). Encapsulated cells also provide greater benefits than mobile cells due to their increased stability, usability, etc.

Investigation of the Mechanism of Cr(VI) % Resistance

When DAS1 was grown with and without Cr(VI), it was noticed that there was a lag phase when the metal was present in the medium, indicating that chromium tolerance

Table 5: Cr(VI) reduction by mobile and immobile cells of DAS1.

Sl. No.	Sample	Reduction % of Cr(VI) after 48 hrs.
1.	Mobile bacteria	85
2.	Immobile bacteria	90.4

was not constitutive but rather inducible. Moreover, after treatment with Na2EDTA, DAS1 maintained its resistance to chromium salt, demonstrating that the impermeability of the outer cell membrane was not the cause of the metal tolerance.

CONCLUSION

At pH 8 for 200 mg.L^{-1} of Cr(VI), *Bacillus licheniformis* DAS1 showed maximum growth, with a specific growth rate (h-1) of 0.062125 h-1 and a bioreduction potential of 85%. Yet, when Bacillus licheniformis DAS1 was bound in calcium alginate beads, bioreduction potential rose to 90.4%. In addition to increasing the specific growth rate (h^{-1}) to 0.082863h-1 and bioreduction potential to 96.02%, glucose served as a metabolic substrate.

The Monod equation was used to analyze the impact of glucose on *Bacillus licheniformis* DAS1 growth, and the line weaver-Burk plot was used to compute ks and max, which were found to be 0.434 g.L^{-1} and 0.090h^{-1}, respectively. 99% of Cr(VI) was converted to Cr using a 1:1 mixture of DAS I and DAS2 cultures (III). *Bacillus licheniformis* DAS1's resistance to Cr(VI) was not caused by the permeability of the cell membrane, and the enzyme activity was inductive rather than constitutive.

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