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Evaluation of Bioremediation Potential of Two Commercial Probiotics for Cr (VI): An *In vitro* Study

Pragya Goyal[1], Pranoti Belapurkar[1]* and Anand Kar[2]

[1]Department of Biosciences, Acropolis Institute, Mangliya, Indore, Madhya Pradesh, India.
[2]School of Life Sciences, Devi Ahilya Vishwavidyalaya, Indore, Madhya Pradesh, India.

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Chromium, in its hexavalent form (Cr (VI)), is a highly toxic and a carcinogenic heavy metal, which is released in the environment largely due to anthropogenic activities. Studies have reported that microorganisms especially probiotics may have the potential to reduce its toxicity under in vitro as well as in vivo conditions. The aim of this study was to assess the effects of various factors on bioremediation potential of two probiotic species of genus Bacillus, B. coagulans and B. clausii for Cr (VI). The factors assessed were initial Cr (VI) concentration, temperature, pH and contact duration. Both organisms showed an exceptionally high Cr (VI) reducing capability from the surrounding media. B. coagulans showed maximum reduction of Cr (VI) at 8 ppm concentration; temperature 40oC; pH 9 and contact duration 48 hrs while for B. clausii these parameters were optimized to be 8 ppm of Cr (VI) concentration, temperature 30oC, pH 7 and contact duration 48 hrs. These results also indicated that the probable strategies adopted by the test microorganisms for bioremediation of Cr (VI) are biosorption and bioaccumulation. The observations were highly promising and therefore, B. coagulans and B. clausii appear to be ideal candidates for potential bioremediation of Cr (VI), in vivo.

Keywords: Bioremediation; Bacillus clausii; Bacillus coagulans; Cr (VI); Probiotic.

Chromium occurs in Earth's crust in valencies ranging from + 2 to + 6 and is one of the heavy metals which does not exist in nature as an element[1]. Out of these valencies, Cr (III) is the most common one followed by Cr (VI)[2]. Various industries like tannery, metallurgy and stainless steel have led to an increase in chromium concentration in the environment, where it is found in Cr (VI) form[3] and has been classified as a carcinogen[3,4].

Cr (VI) persists in the environment and gets transferred either abiotically (via soil, air or water) or biotically (commonly by food) to reach various consumers, including humans. When humans consume food and water laden with Cr (VI), various toxic effects like anemia, stomach ulcer and damage to male reproductive system are observed. It also affects body systems like cardiovascular, hepatic and nervous system and can even cause death[3].

Bacterial species have shown exceptional capacity of bioremediation of toxic compounds like, pesticides[5] and heavy metals[6-9]. They use methods like biosorption, bioaccumulation, efflux, transformation of toxic valency to non-toxic valency, use of metal chelating proteins etc., for detoxification of heavy metals present in the surroundings[10-12]. In this regard, probiotic

*Corresponding author E-mail: pranotibelapurkar@acropolis.in

microorganisms have proven themselves, very useful in bioremediation of heavy metals under *in vitro*[13], as well as *in vivo* conditions[14,15].

Probiotics have been defined as live microorganisms, which when administered in adequate amount, confer health benefits to the host[16]. One of the predominant genus involved in bioremediation of heavy metals is genus *Bacillus*. Probiotic species of this genus have proven to be highly effective in removal and detoxification of heavy metals[17,18] by the methods mentioned above.

Earlier our published works have established that *Bacillus coagulans* and *Bacillus clausii*, have the capacity to tolerate high concentrations of Cr (VI)[6,9]. Various factors like initial heavy metal concentration, temperature, pH and contact time are known to affect the heavy metal sequestration capacity of bacterial cells[19]. The present study attempts to evaluate if all affect bioremediation potential of *B. coagulans* and *B. clausii* for bioremediation of Cr (VI) or not.

MATERIALS AND METHODS

The organisms selected for the study were *Bacillus coagulans* and *Bacillus clausii*. *B. coagulans* is available as a spore tablet Sporlac®-DS, marketed by Sanzyme (P) Ltd., Dehradun, Uttarakhand, India and *B. clausii* is available as a spore suspension Enterogermina® marketed by Sanofi Synthelabo Pvt Ltd., Powai, Mumbai, India. Sporlac®-DS contains 0.12 billion spores of *B. coagulans*/ tablet, while Enterogermina® contains 2 billion spores of *B. clausii*/ ml. Potassium dichromate ($K_2Cr_2O_7$) was used as source of test heavy metal Cr (VI).

Effect of initial heavy metal concentration

The test organisms (*B. coagulans* and *B. clausii*) were inoculated in nutrient broth supplemented with two-fold concentration of Cr (VI) ranging from 1-32 ppm. For this, inoculum was prepared by germinating the spores in nutrient broth for 24 hours. All the tubes were incubated at 37°C for 24 hours, followed by centrifugation of the broth at 10,000 rpm for 10 minutes. The supernatant was collected for estimation of residual Cr (VI) concentration by methodology of Mala et al. (2015) [20], with some modifications. Reaction mixture for the estimation was prepared by adding 5 ml of supernatant (NB containing Cr (VI)), 0.5 ml distilled water (DW), 0.5 ml H_2SO_4 and 2 ml of 0.5% diphenyl, carbazide (DPC) in acetone. This was allowed to stand for 15 minutes at room temperature for development of pink colour. Optical density (OD) was measured in UV-Vis spectrophotometer at 540 nm against a blank. The values were expressed as percentage reduction of Cr (VI), using the following equation[21].

$$\% \text{ reduction} = (C_i - C) / C_i \times 100$$

where,

C_i is the initial concentration of the metal in the solution (ppm) and

C is the concentration of metal in the solution (ppm) after a specified time (hrs)

The concentrations at which, both the test organisms, respectively showed maximum percentage reduction of Cr (VI) were used for all further experiments.

Effect of temperature

For incubation, different temperatures were selected for the study- 25°C, 30°C, 35°C and 40°C. *B. coagulans* and *B. clausii* were inoculated in NB tubes containing specific concentration of Cr (VI) (as obtained previously) and incubated at different temperature. After 24 hours, residual Cr (VI) was estimated as previously described and expressed as percentage reduction (Eq. 1). The temperature at which maximum percentage reduction of Cr (VI) was observed for *B. coagulans* and *B. clausii,* respectively was the optimum temperature.

Effect of pH

Five pH values selected for the study were pH 5, 6, 7, 8 and 9. *B. coagulans* and *B. clausii* were inoculated in NB tubes of specific pH value containing specific concentration of Cr (VI) (as obtained previously). These tubes were incubated at 37°C for 24 hours and residual Cr (VI) concentration was estimated as described previously. The result was expressed as percentage reduction (Eq. 1). The pH at which maximum percentage reduction of Cr (VI) was observed for *B. coagulans* and *B. clausii*, respectively was the optimum pH value.

Effect of contact duration

B. coagulans and *B. clausii* were inoculated in NB tubes with specific concentration of Cr (VI) (as obtained previously), and incubated

at 37°C degrees for 24, 48, 72 and 96 hr. The residual concentration of Cr (VI) after each contact duration was estimated as described previously and was expressed as a percentage reduction of Cr (VI). The duration at which maximum reduction of Cr (VI) was observed by the test organisms was the optimum contact duration.

Statistical analysis

All the above experiments were performed in triplicate and the values have been expressed as mean ± sem (standard error of means).

RESULTS AND DISCUSSION

Effect of initial Cr (VI) concentration

B. coagulans and *B. clausii*, both showed maximum reduction at 8 ppm. They reduced 18.603% (Fig. 1) and 44.043% (Fig. 2) of Cr (VI) respectively at this concentration. Similar trends have been obtained for *Bacillus* sp. FY1[22], *B. cereus*[23] and *B. subtilis*[24], where 100% reduction was observed at 200 mg L^{-1}, 90% at 200 ppm and 60% at 200 ppm, respectively.

Bacillus is a gram-positive microorganism and has a negatively charged cell wall (due to the presence of teichoic acid, teichuronic acid, carboxyl and amino groups)[25]. These negatively charged functional groups bind metal cations and help in reducing heavy metal concentration from the surroundings[26,27]. As observed in the experiment, the decrease in percentage reduction of Cr (VI) after the optimum concentration is possibly due to decrease in availability of free binding sites. These sites become saturated with increase in concentration of heavy metals[28], hence leading to reduced Cr (VI) removal capacity of the test microorganisms.

Another potential explanation for this phenomenon could be the toxicity of Cr (VI). After optimum concentration of Cr (VI), a decline in the ability of the test organisms to reduce Cr (VI) has been observed up to 32 ppm. This decrease may stem from the toxicity of Cr (VI) at elevated concentrations[29], resulting in a likely reduction in cell numbers and consequently diminishing the availability of attachment sites for Cr (VI) on the bacterial surface.

In addition to utilizing binding sites on the bacterial surface, *Bacillus* sp. also produces the enzyme chromate reductase, which converts toxic Cr (VI) to non-toxic Cr (III)[20]. At higher Cr (VI) concentrations, there is a possibility of enzyme deactivation, leading to a decrease in the capacity of *B. coagulans* and *B. clausii* to remove Cr (VI) from the surrounding medium[30], as observed in the experiment.

Effect of temperature

For reduction in concentration of Cr (VI), optimum temperature for the test organisms were obtained above 30°C, i.e. for *B. coagulans* was observed at 40°C (Fig. 3) while for *B. clausii* it was at 30°C (Fig. 4) where 85.09% and 81.59% reduction of Cr (VI) was observed respectively. Similar findings were observed in the bioremediation of Cr (VI) using environmental strains of the *Bacillus* genus. For instance, *Bacillus* FY1 demonstrated an 88.5% reduction in Cr (VI) at 30°C and comparatively less reduction was found at 25°C and 40°C [22]. Likewise, research on *B. cereus* indicated a 100%[31] and 66.4%[32] reduction in Cr (VI) at 40°C. In two separate studies, temperatures above 30°C, specifically 35°C, were identified as the optimal temperature for Cr (VI) reduction by *B. subtilis*[33] and *B. thuringiensis*[34], resulting in reductions of 93.6% and 85.23%, respectively.

Temperature affects microbial biomass and metal- microbe interaction and in turn influences the bioremediation potential of bacteria. Changes in temperature causes change in cell wall configuration, stability of metal microbe complex and ionization of groups on cell wall[35,36]. In this experiment, it was observed that with rise in temperature, rate of reduction in concentration of Cr (VI) increased. This could be due to increase in pore size of bacterial surface and rate of heavy metal diffusion[37-39]. This continued till the optimum temperature was reached. However, beyond this temperature, factors such as weak metal binding, change in cellular metabolism[40] and deactivation of bacterial cell wall[41-43] could have led to decrease in reduction capacity of the test microorganisms for Cr (VI). An additional factor for this observation might be alterations in enzyme ionization rates and modifications in the structure of proteins such as chromate reductase[44], which could potentially lead to its denaturation[40].

Effect of pH

At pH 9, it was observed that *B. coagulans* reduced 100% of Cr (VI) (Fig. 5), while maximum

reduction (82.167%) of Cr (VI) by *B. clausii* was observed at pH 7 (Fig. 6). From the experiment, it was also observed that percentage reduction was low at acidic pH, while it increased till it reached optimum pH i.e. pH 9 for *B. coagulans* and pH 7 for *B. clausii*. Similar result has been obtained for *B. cereus* and *B. thuringiensis* where 100%[31] and 86.42%[34] reduction in Cr (VI) was found, respectively at pH 7. Another study has reported the ability of *B. subtilis* to reduce 96% Cr (VI) at pH 9[45]. It has been postulated that the reason for this is the cell wall of *Bacillus*, which has a net negative charge[13,46]. This is due to the influence of pH on the number of binding sites available for cations like Cr (VI) and the speciation of metals[47,48]. The affinity of cationic species for the functional groups on the cell surface is significantly influenced by the pH of the solution. When the pH of the surrounding medium is low, concentration of hydronium ions (H_3O^+) is high. These ions have a strong affinity for the negatively charged functional groups present on the cell wall and thus compete with positively charged metal ions like Cr (VI) ions also present in the surrounding medium. This leads to a decreased reduction capacity of the test microorganisms. However, as the pH increases, H_3O^+ concentration decreases, degree of ionization of functional group such as amino, carboxyl, imidazole and phosphate

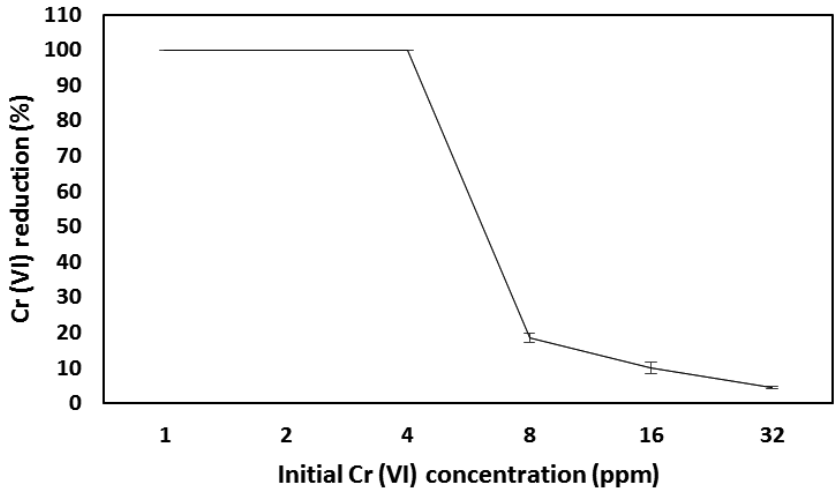

Fig. 1. Effect of varying initial Cr (VI) concentration on reduction (%) of Cr (VI) by *B. coagulans*

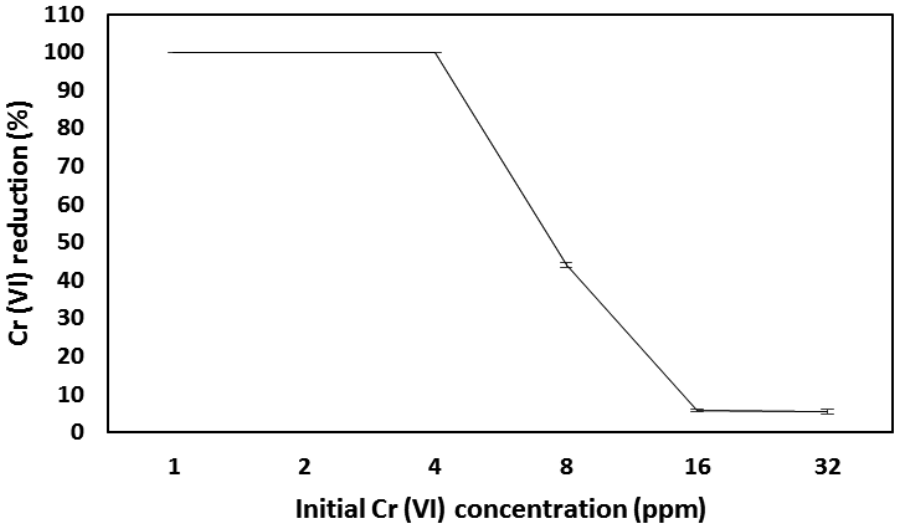

Fig. 2. Effect of varying initial Cr (VI) concentrations on reduction (%) of Cr (VI) by *B. clausii*

increases and they get more exposed[49,50]. This probably led to increase in Cr (VI) reduction ability of *B. coagulans* and *B. clausii* at higher pH values.

When the functional groups were removed from the cell wall, a drastic decrease in metal uptake capacity was observed[13], thus highlighting the importance of these functional groups in heavy metal bioremediation.

pH levels also impact the bioremediation capabilities of *Bacillus* sp., indirectly. This can happen when deviations from the optimal pH can alter the structure and activity of chromate reductase[31]. Therefore, it is crucial for *Bacillus* sp. to maintain the optimal pH to achieve maximum reduction in the concentration of Cr (VI).

Effect of contact duration

Both the test organisms showed 100% reduction of Cr (VI) at 48 hr (Fig. 7, 8) and an increase in time did not change the reduction capability of the test microorganisms. This is probably due to the phenomenon where the functional groups present in the cell membrane

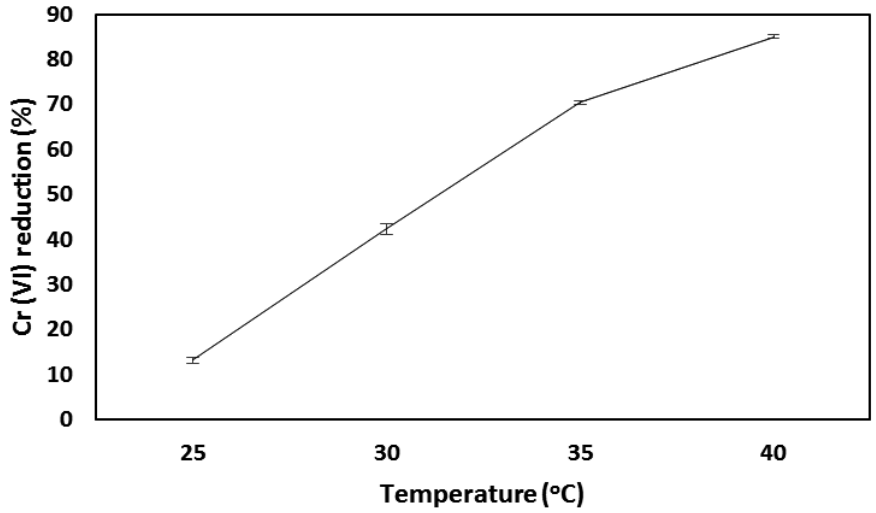

Fig. 3. Effect of varying temperature on reduction (%) of Cr (VI) by *B. coagulans*

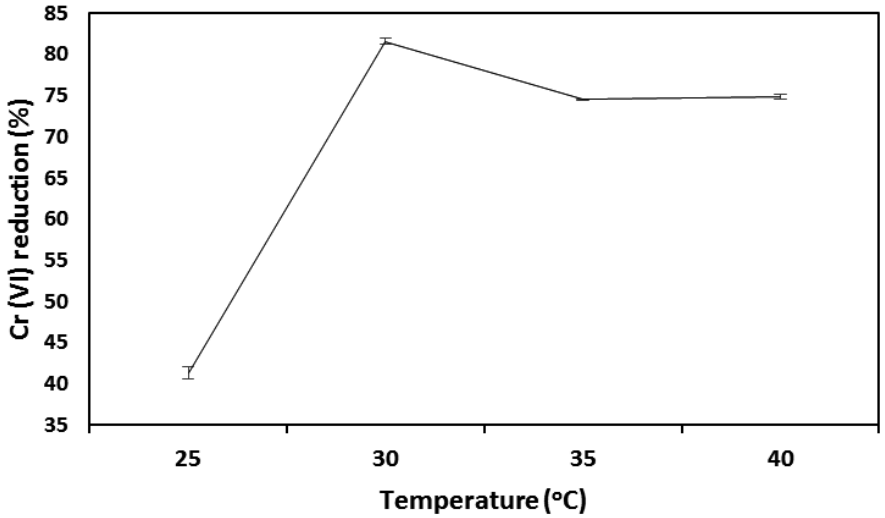

Fig. 4. Effect of varying temperature on reduction (%) of Cr (VI) by *B. clausii*

of the bacteria become saturated with Cr (VI) and restrict the microbial cells for further uptake of metal ions[51]. Studies conducted by various researchers have reported that *B. cereus* reduced 100% of available Cr (VI)[31] and *B. thuringiensis* reduced 77.3% Cr (VI)[34] after 72 hrs and 48 hrs, respectively. It has also been reported that in the initial phase, the rate of reduction was faster, but decreased with increase in contact duration[21]. This suggests that there are two phases i.e. primary and secondary. Primary phase is faster, while secondary phase is slower[21]. The probable reasons for faster initial reduction in primary phase are a steep concentration gradient and availability of more empty binding sites[39,52,53]. The subsequent slower phase may be due to (i) limited availability of active sites, and/or (ii) a reduction in the concentration gradient.[21,51]. As the adsorption process continues, the sorbed solute begins to desorb back into the solution and continues till a state of equilibrium. At this point, the rates of adsorption and desorption balance out, resulting in no further net adsorption[54].

Culture age also plays an important role. When the culture is mid-log to log, cells are more metabolically active and produce more enzymes and proteins responsible for heavy metal uptake[36] like chromate reductase. This suggests that though the initial mechanism of bioremediation

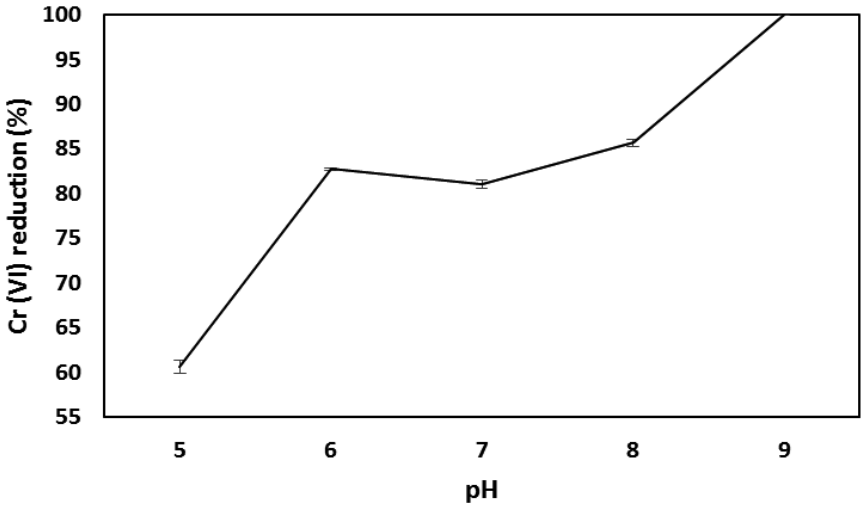

Fig. 5. Effect of different pH on reduction (%) of Cr (VI) by *B. coagulans*

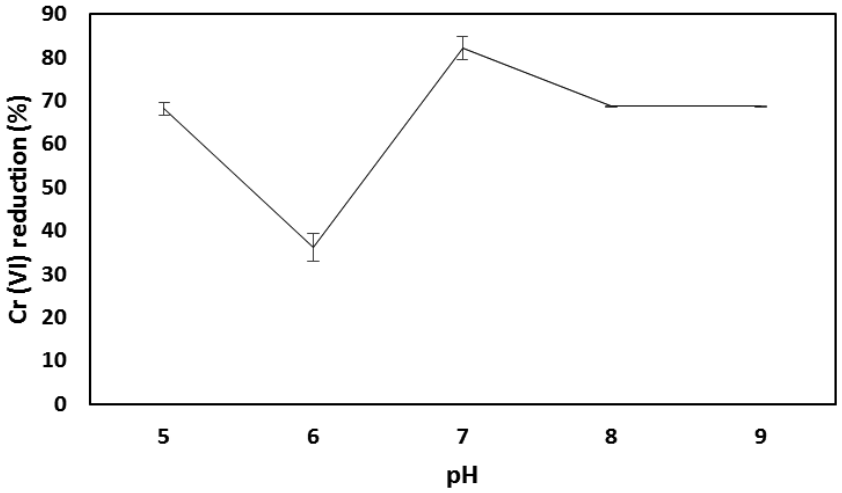

Fig. 6. Effect of different pH on reduction (%) of Cr (VI) by *B. clausii*

Table 1. Percentage reduction of Cr (VI) at optimum values of the parameters studied

S. no.	Test Microorganism	% Cr (VI) reduction at optimum Cr (VI) Conc. (ppm)	% Cr (VI) reduction at optimum Temperature (°C)	% Cr (VI) reduction at optimum pH	% Cr (VI) reduction at optimum contact duration (Hrs)
1.	*Bacillus coagulans*	18.60±1.38% at 8 ppm	85.09±0.28% at 40°C	100.07±0.0% at pH 9	100±0.0% at 48 hrs
2.	*Bacillus clausii*	44.04±0.63% at 8 ppm	81.59±0.37% at 30°C	82.17±2.71% at pH 7	100.0±0.0% at 48 hrs

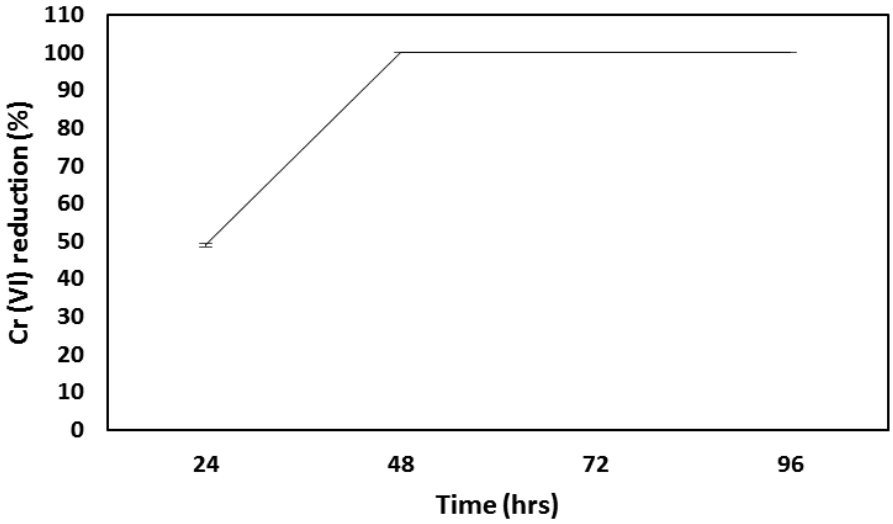

Fig. 7. Effect of different contact duration on reduction (%) of Cr (VI) by *B. coagulans*

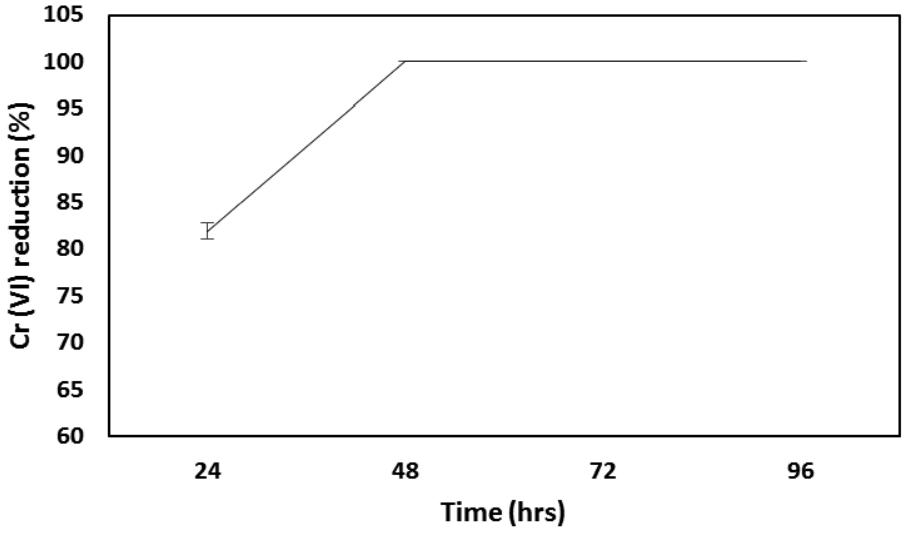

Fig. 8. Effect of different contact duration on reduction (%) of Cr (VI) by *B. clausii*

is biosorption in the primary phase, later it is bioaccumulation in secondary phase[55,56].

The results obtained from the study, as summarized in Table 1, strongly suggest that the test probiotics probably use mechanisms of biosorption and bioaccumulation for bioremediation of Cr (VI). However, studies need to be performed to verify this. It can be concluded that *B. coagulans* and *B. clausii* have the potential for *in vivo* bioremediation Cr (VI), and can be used for societal welfare by reducing the toxicity of Cr (VI) in the human gut.

CONCLUSION

Probiotics have the potential to reduce the concentrations of heavy metals from surrounding media, under *in vitro* as well as *in vivo* conditions. Chromium (VI), a major contaminant of soil and water resources, is a toxic heavy metal and a recognized carcinogen. When the effects of various factors were studied to assess the bioremediation potential of *B. coagulans* and *B. clausii* for Cr (VI), it was observed that both the test organisms showed an exceptionally high Cr (VI) reducing capability from the surrounding media. The optimum values of pH, temperature and contact duration for Cr (VI) bioremediation were in corroboration with its probiotic potential. The probable mechanisms of bioremediation of Cr (VI) elucidated from the above results were biosorption and bioaccumulation. Hence, it can be concluded that both the test microorganisms are promising candidates for bioremediation of Cr (VI), *in vivo*.

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Conflict of interest

The authors certify that we have no conflict of interest.

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Authors' contribution

Dr. Pragya Goyal: Data collection, analysis and interpretation of results, draft manuscript preparation. Dr. Pranoti Belapurkar: Study conception and design, analysis and interpretation of results, Dr. Anand Kar: Study conception and design. All the authors reviewed the results and approved the final version of the manuscript

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

Ethical approval statement

NA.

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