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Research Article

Kinetic Studies and Bioremediation Potential of Chromate Reductase-Characterized *Aspergillus flavus* from Tannery Effluent

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ABSTRACT

Chromium is among the major environmental pollutants endangering the health of all living things, including humans. Conventional methods for chromium detoxification are expensive, so bioremediation using fungi as potential agents for the detoxification of chromium is justified. This study aims to investigate chromium-resistant fungi capable of reducing or transforming Cr (VI) to less toxic Cr (III) from tannery effluents. The fungi were isolated from tannery wastewater and identified using 16S rRNA gene sequencing. Chromate reductase was isolated from pure fungal isolate and purified using molecular weight estimation and gel filtration chromatography. *Aspergillus flavus*, in an interesting turn of events, demonstrated 97% chromate reduction. The chromate reductase from *Aspergillus flavus* has K_M of 0.015 mM and a Vmax of 0.25 mol/min. Furthermore, the enzyme's activity was constant at its peak at pH 7.0 and 50 ºC, with a single band displaying a molecular weight of 44 kDa for a pure protein. The data suggest that *A. flavus* can be a good candidate for the detoxification of Cr (VI) in industrial effluents. The efficiency of which can be attributed to the action of chromate reductase in the *A. flavus*.

Keywords: *Chromate reductase, Aspergillus flavus, Tannery effluent, Kinetic properties, Enzyme activity*

INTRODUCTION

Globally, there is a growing concern over environmental pollution (such as heavy metals) caused by the emission of hazardous wastes from mining operations and the leather industry (Abdulsalam *et al*., 2016). Chromium (Cr), a naturally occurring transition metal, is extensively used in the electroplating and manufacturing of metals, paints, ceramics, and the tanning of leather (Singh *et al*., 2021).

Many tannery effluents are often discharged into the environment without treatment, where they seep into the groundwater and contaminate it with hazardous metals such as Cr (VI) and Fe $(III)^{+}$. Cr (III), NaCl, and NaS are among the few contaminants present in tannery effluents (Habila *et al*., 2020; Igiri *et al*., 2018).

Effluent treatment would aid in preventing toxins from entering the food chain and contaminating drinking water (Pang *et al*., 2010). The biological methods for treating tannery effluents (e.g., biosorption or bioaccumulation approaches) may offer appealing substitutes to current

technologies, such as the extremely expensive electrochemical method. Microbial biomass can function as efficient metal scavengers both through bioaccumulation and biosorption (Okoduwa *et al*., 2017). Direct use of microorganisms with specific catabolic capabilities and/or their products, such as enzymes and biosurfactants, is a novel way to enhance and increase the effectiveness of remediation operations (Gauje *et al.,* 2022; Igiri *et al.,* 2018). Fungi can tolerate large amounts of heavy metals because their cell walls are adept at absorbing metals (Pradhan *et al.,* 2017). Chitosan, glucosamine, and chitin are the three most significant components of the fungal cell wall, with glucosamine serving as the primary metal-binding component (Elahi *et al.,* 2020). For the remediation of Cr (VI), the *Aspergillus flavus* CR500 strain has been utilized in addition to other techniques (Kumar and Dwivedi, 2019).

According to reports, the *A. flavus* isolate TERIBR1 from chromium-contaminated tannery sludge can thrive in culture conditions containing up to 250 mg/l of chromium (Singh *et al.,* 2019). Enzymes are often required for the metabolism of chromium by fungi, and the numerous methods may include active metal uptake, intracellular and extracellular precipitation, and redox reactions (Akcil *et al.,* 2015). Although, it has been reported that *A. flavus* and other species can remove Cr (VI) from contaminated environments (Bennett *et al.,* 2013; Kandasamy *et al.,* 2021a, 2021b; Whangchai *et al*., 2021). Numerous chromate reductases have been isolated from various microbial sources (Robins *et al.,* 2013; Sallau *et al.,* 2014; Sandanamala *et al.,* 2015). The purpose of this study was to characterise chromate reductase purified from fungi isolated from industrial tannery effluent and determine whether the isolated enzyme (chromate reductase) can effectively convert harmful Cr (VI) to less toxic Cr (III).

MATERIALS AND METHODS

Isolation and Characterization of Fungi from Tannery Effluent

The isolation of fungi from a tannery wastewater sample was carried out by the method of Sugasini *et al.* (2015). Exactly 10 ml of the sample was placed in a test tube and centrifuged at 448 x *g* for 5 minutes. The supernatant was decanted, and 0.1 ml of the filtrate was sprayed on Potato Dextrose Agar (PDA) plates previously sterilised at 121ºC for 15 minutes.

The sprayed PDA plates were incubated at room temperature for 5 days and observed for fungus growth. Mixed colonies were observed, and the prominent ones were subcultured into fresh PDA plates in order to obtain pure culture. The plates were also incubated at room temperature for five days. After the incubation period, the plates were observed for the growth of pure fungal isolates. A smear of each pure fungal isolate was done on a glass slide using lactophenol cotton blue and observed under the microscope.

Bioremediation of Tannery Effluent for Chromate Reduction

The method of Aneez *et al.* (2011) was used to bioremediate tannery effluent by employing fungal isolates for chromate remediation. First, the pure fungal isolates (PFI) were diluted with 5 ml of Tween 80 each before being inoculated into the experimental mixture, and the number of cells per ml was calculated using a hemocytometer. 1.365 x 106 cells/mL was used as the unit of measurement for the inoculation. Then, 50 ml of the tannery wastewater sample was transferred to a conical flask with 50 ml of prepared media, and 3 ml of the diluted pure fungal isolate was added to the conical flask. The flask was put in an orbital shaker for 72 hours at 30 ºC, spinning at 250 rpm. Then, a heavy metal (chromium) examination of the tannery effluents was done. The bioremediation potentials of the fungi were determined by comparing the levels of chromium before and after the experiment.

Identification by 16S rRNA Gene Sequence

DNA was extracted using the Accuprep Genomic DNA Extraction Kit (K-3032) following the manufacturer's instructions. The fungal mycelia were centrifuged at 300 x *g* for 5 minutes. The supernatant was carefully discarded without disturbing the pellets. The pellets were resuspended in 200 µl of 1 x phosphate buffered saline (PBS). Then 20 µl and 10 µl of proteinase K and RNase A were added, respectively, mixed thoroughly, and incubated at room temperature for 2 minutes. After the incubation period, 200 µl of GB buffer was added, mixed with vortex, and incubated at 60 ºC for 10 minutes.

After the incubation period, 400µl of absolute ethanol was added and mixed by pipetting. The lysate was then carefully transferred into a binding column tube (fitted in a collection tube) and centrifuged at 72 x *g* for 1 minute. The solution in the collection tube was discarded, and the collection tube was reused. Then, 500 µl of washing buffer (WA1 Buffer) was added and centrifuged at 7168 x *g* for one minute to wash the genomic DNA molecules.

The solution collected was discarded, and the tube was reused. Another 500 µl of washing buffer (W2 Buffer) was added and centrifuged at 7168 x *g* for one minute to rewash the banded DNA, and the solution collected was discarded. The tube was centrifuged once more at 18,928 x *g* for 1 minute to completely remove ethanol and ensure that there was no droplet clinging to the bottom of the binding column

tube. The binding column tube was then transferred to a new 1.5 ml tube for elution by adding 50-200 µl of elution buffer (EA Buffer) to the binding column tube, allowed to stand for 1 minute at room temperature, and then centrifuged at 7168 x *g* for 1 minute to elute the extracted DNA.

The extracted DNA from the fungal isolate was amplified using Internal Transcribed Spacer (ITS) primers, viz. ITS 1 Forward: 5' TCCGTAGGTGAACCTGCGG 3', ITS 4 Reverse: 5' TCCTCCGCTTATTGATATGC 3' designed by Bioneer Inc., USA.

The PCR reactions were carried out in 20 µl reaction volumes. DNA extract (2 µl) was mixed with 2 µl primers and 16µl of PCR-grade deionized water, making up a 20 µl reaction mixture. The reaction mixture was placed in a PCR tube. The PCR conditions were set as follows for the fungal isolate DNA amplification: an initial denaturation step was set at 94°C for 3 minutes, followed by denaturation at 94^0C for 40 seconds, annealing at 54^0C for 40 seconds and extension at 72^0C for 40 seconds. Thirty-five cycles were carried out with the final extension step at 72^0C for 10 minutes each.

The PCR product was subjected to gel electrophoresis on a 1.0% agarose gel along with a 100-bp DNA ladder in 50 mM Tris-acetate-EDTA buffer pH 8.2 at 100 volts for 30 minutes. Gel was visualised in a gel documentation system.
The amplified PCR product was washed and then subjected to DNA sequencing, and the DNA sequence obtained was subjected to a homology search using the BLAST of the National Centre for Biotechnology Information (NCBI) database.

Production and Extraction of Chromate Reductase from *Aspergillus flavus*

Chromate reductase from pure a fungal isolate was extracted by the technique described by Sallau *et al.* (2014). Fungal cells were grown in a submerged culture medium for chromate reductase production. The fresh media was then aliquoted into four (4) sterile 250 ml Erlenmeyer flasks. Spores of the fungi were harvested from a 7-day-old culture slant by washing with 0.1% Tween 80 (sterilised by autoclaving at 121^0C for 15 minutes), and inoculated into the Erlenmeyer flask containing the fresh media.

The fungi were grown in these flasks on an orbit shaker at 250 rpm and a temperature of 30^0C for 120 hours. The fungi were then harvested by filtering the contents of the flasks containing the fungal mycelia using Whatman No. 1 filter paper, and the mycelia were then washed with 50 mM Tris-HCl buffer pH 7.0. The mycelia harvested were then homogenised using a tissue homogenizer with 120ml of chilled 50 mM Tris-HCl buffer pH 7.0 at a 30-second interval until the cells became completely homogenised.

The homogenate was then centrifuged at 10,000 x *g* for 15 minutes to remove cellular debris and obtain a clear supernatant, which served as the chromate reductase crude extract from the fungi. Aprotinin was also added to a final concentration of 1 microgram % (w/v) to stabilise the enzyme.

Chromate Reductase Purification

Chromate reductase was purified from *A. flavus* according to the method previously described by Sallau *et al.* (2014). In a nutshell, at each stage of the purification, the activity of the enzyme chromate reductase as well as the protein concentration were measured. For the separation of ammonium sulphate, crude chromate reductase extract was precipitated with 35%-90% saturation of solid ammonium sulfate at 4^0C overnight. The precipitate was centrifuged at 4,000 x g for five minutes before being dissolved in a solution of 50 mM Tris-HCl buffer at pH 7.0 in 5 ml. Both the protein content and chromate reductase activity of the dissolved precipitate were measured. A 50 mM Tris-HCl buffer with a pH of 7.0 was used to dialyze dissolved crude enzyme for 24 hours at 4 ^{0}C before the buffer was changed.

The crude chromate reductase was filtered using a gel filtration column with settled Sephadex G-75 that had been pre-equilibrated with 50 mM Tris-HCl buffer pH 7.0. The column was then eluted with the same buffer, collecting 30 fractions at a rate of 0.25 ml/min while chromate reductase activity and total protein concentration were measured. The greatest chromate reductase activity fractions were combined for ion exchange chromatography in a column. In order to collect 30 fractions (each 5.0 ml in volume), the column was loaded with 3.0 ml of the chromate reductase active fraction and continuously eluted with NaCl solution (at a gradient of 0.00 M to 1.00 M).

Tests for Chromate Reductase Activity

Chromate reductase activity was assayed according to the method of Park et al. (2000), as slightly modified by Sallau *et al.* (2014). The activity was examined during a 60-minute incubation of a reaction mixture at 30 ^{0}C. 100 ml of sample (enzyme extract), 300 ml of 0.05 mM potassium dichromate (substrate), 300 l of 0.1 mM NADH, and 300 l of 50 mM Tris-HCl buffer pH 7.0 made up the reaction mixture, which had a final volume of 1.0 ml. The reaction was stopped with the addition of one (1.0 ml) solution of 0.2% 1,5-diphenylcarbazide, which was then let stand for 10 minutes. Using a spectrophotometer, the absorbance at 540 nm was measured against a control sample. The amount of enzyme needed to convert one mole of chromate VI to chromate III

under specific assay conditions was determined by one unit of chromate reductase activity.

Determination of Total Protein Concentration

The Biuret test was used to determine the total protein content. One (1.0) ml of the protein sample was added into a test tube containing 4.0 ml of the biuret reagent. The test tube was incubated at room temperature for 30 minutes, and absorbance was taken at 540 nm using a spectrophotometer. Protein concentration was deduced from the bovine serum albumin (BSA) standard curve.

Kinetic Studies of Chromate Reductase obtained from *Aspergillus flavus*

Initial velocity experiments were performed on the partly purified chromate reductase, and activity at various substrate concentrations (0.01–0.05 mM) was determined (potassium dichromate). Lineweaver-Burk plots were used to plot the reciprocal relationship between enzyme activity and reciprocal substrate concentrations, and KM and Vmax values were calculated.

Effect of pH on the activity of chromate reductase obtained from *Aspergillus flavus*

The activity of the partially purified chromate reductase was determined as a function of pH using acetate buffer pH 3.5-6.5 and Tris-HCl buffer at pH 7.0-8.0). The plot of pH values and enzyme activity was prepared to determine the optimum pH.

Effect of temperature on chromate reductase activity obtained from *Aspergillus flavus*

The activity of the partially purified chromate reductase was determined as a function of temperature. The temperature-dependent studies were conducted at different temperatures, and a plot of the enzyme activity against the temperature range was prepared to determine the optimum temperature for the enzyme's activity.

Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis (SDS PAGE)

The partially purified enzyme sample (35 µl), crude enzyme, and molecular weight marker were heated at 95 °C for 5 minutes in an Eppendorf tube with 0.5 ml of the treatment buffer (1% SDS, 1% mercaptoethanol, and 0.1 M phosphate buffer, pH 6.8). 10 lml of glycerol and 7.5 µl of bromophenol blue as the loading dye were added to each tube. Trisglycine buffer (pH of 6.8) was used for two hours of electrophoresis. Following electrophoresis, the gel was taken out and stained for two hours with Comassie Brilliant Blue. It was then rinsed and destained in 7% acetic vinegar, and bands were checked for using the bands of the molecular weight marker, the molecular weight of the isolated enzyme was calculated.

RESULTS

Enumeration of Cr (VI) tolerant fungi

The third day of the bioremediation procedure revealed an increase in *A. flavus* biomass and a decrease in the Cr (VI) content. The outcomes showed that the isolate PFI2 had notable Cr (VI) reduction activity at 97% in 72 hours (Figure 1).

Molecular identification of the most active Cr (VI) pure fungal isolate (PFI)

The 16S rRNA gene sequencing techniques were employed to identify PFI2 fungi cells. The details of the sequence are available as supplementary data. According to the NCBI-BLAST, the fungi cell was identified as *A. flavus* and received accession number MK494014.1

Chromium reduction potential of *Aspergillus flavus*

At a concentration of 7.36 mg/L over the course of three days, the bioremediation capacity of the local Cr (VI)-tolerant *A. flavus* was examined. The reduction was directly related to the number of days that the bioremediation process took to complete in the Cr (VI) lowering procedure. The third day of the bioremediation procedure saw an increase in *A. flavus* biomass and a decrease in the Cr (VI) content. The outcomes showed that the isolate PFI2 had notable Cr (VI) reduction activity at 97% in 72 hours (Figure 1). According to the investigation on Cr (VI) reduction, the PFI2 fungus isolate exhibited remarkable Cr (VI) reduction. The final two strains provided significant levels of resistance to Cr as well (VI).

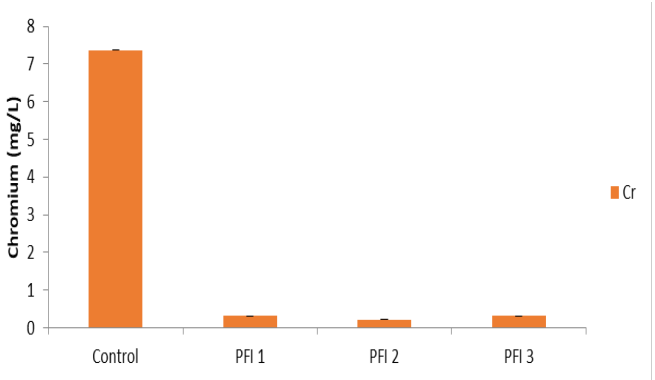

Figure 1. Chromium Concentration of Tannery Effluent Before and After 72 Hours of Bioremediation Using Pure Fungal Isolates (PFI)

Purification Profile of chromate reductase from *A. flavus*

The purification of chromate reductase extracted from *A. flavus* revealed high potential for Cr (VI) bioremediation as presented (Table 1). The increase in specific activity of the chromate reductase from *A. flavus* as the purification steps progresses is indicative of the enzyme's increased purity and this suggest the removal of impurities from the crude protein.

The chromate reductase isolated from *A. flavus* moved as a single band (Plate 1) with a molecular weight of 44 kDa on SDS-PAGE, indicating good purity. The highest enzyme activity was found in the pooled eluates (12, 17, 21, 22, 27, 28, and 29 fractions) of the gel filtration chromatography on Sephadex G-75 with Tris-HCl buffer pH 7.0 (Figure 2). The excellent purity of the protein was indicated by fraction 22 of the ion exchange chromatography having chromate reductase activity (Figure 3).

The enzyme exhibited optimum activity at pH 7.0 (Figure 4). This maximum activity recorded at neutral pH may offer the suitability of the enzyme for efficient bioremediation of hexavalent chromium in industrial effluents. Temperature-dependent studies on the activity of the enzyme revealed an optimum temperature for the activity of the enzyme to be 50 ^{0}C as presented (Figure 5).

Table 1. Purification Profile for Chromate Reductase Isolated from *Aspergillus flavus*

Purification Steps	Vol. (ml)	Total Protein (mg)	Total Activity (µmole/min)	Specific Activity (µmole/min/mg)	% Yield	Purification Fold
Crude Enzyme	120.00	0.28	0.82	2.93	100.00	1.00
Ammonium Sulfate Fractionation (35% - 90%)	5.00	0.14	0.48	3.43	58.54	1.17
Gel Filtration Chromatography (Sephadex G-75)	3.00	0.02	0.28	14.00	34.15	4.78

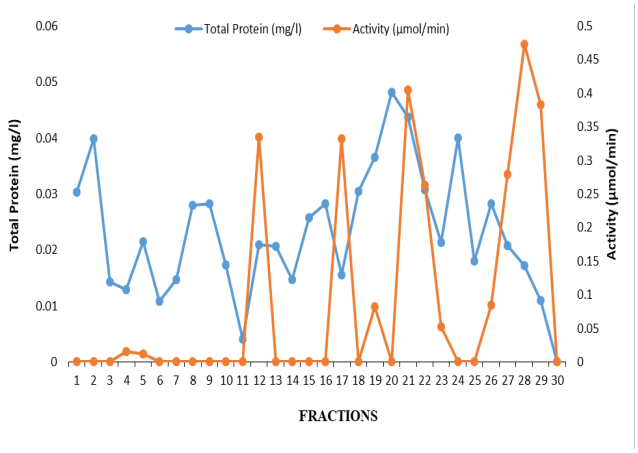

Figure 2. Elution Profile of Chromate Reductase isolated from *Aspergillus flavus* from Sephadex G – 75 Gel Filtration Column.

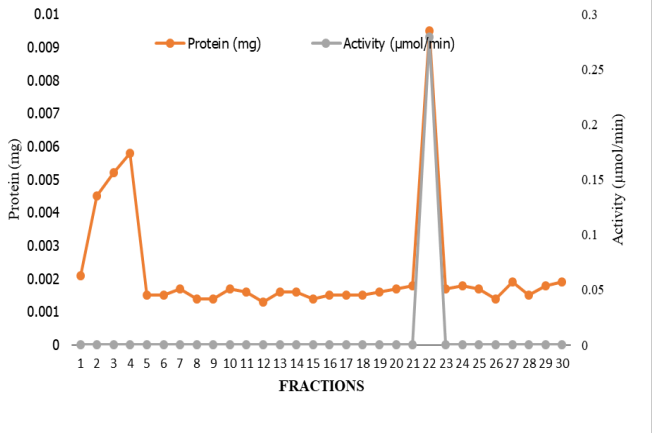

Figure 3. Elution Profile of Chromate Reductase isolated from *Aspergillus flavus* from Ion - Exchange Chromatography (Dowex 50Wx8) Column

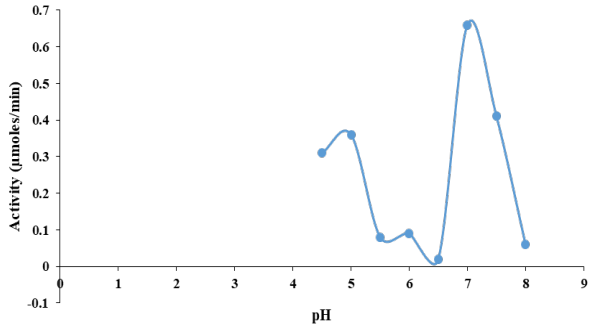

Figure 4. Activity of Chromate Reductase isolated from *Aspergillus flavus* at varying pH

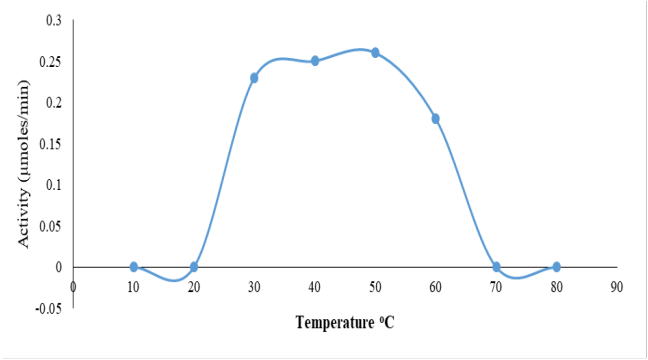

Figure 5. Activity of Chromate Reductase isolated from *Aspergillus flavus* at varying Temperature

Kinetic studies for chromate reductase isolated from *Aspergillus flavus*

Initial velocity studies carried out on the chromate reductase isolated from *A. flavus* using potassium dichromate as substrate revealed a K_M and V_{max} of 0.015 mM and 0.25 µmol/min respectively as described in Figure 6. K_M represents the concentration of the substrate which permits the enzyme to achieve half V_{max}. This means that an enzyme with a high K_M has a low affinity for its substrate and therefore requires a greater concentration of the substrate to achieve V_{max}

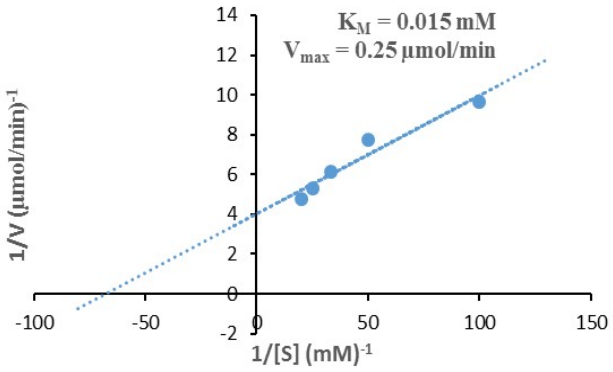

Figure 6. Lineweaver – Burk plot for Chromate Reductase isolated from *Aspergillus flavus*

DISCUSSION

Fungi are potential microbes that can be employed to bioremediate organic and heavy metal toxins. They may also secrete exudates, which act as a source of energy throughout the remediation process (Harms *et al.,* 2011). Due to the existence of cell walls with excellent metal-binding properties, their cells can survive in toxic metal-contaminated areas. All stages of the fungal life cycle produce enzymes from fungus, and these metabolites are sufficient to remediate contaminants (Ryan *et al.,* 2005).

The most hazardous heavy metal is hexavalent chromium, which has a high oxidative potential that results in cell destruction and has mutagenic, carcinogenic, and teratogenic consequences (Tadishetty *et al.,* 2017). For a study on Cr (VI) reduction, a chromium-resistant fungus strain called *A. flavus* obtained from contaminated tannery wastewater was employed. Under optimal conditions of 50^{0}C, 72 hours of incubation, and pH 7.0, the isolated fungal strain significantly decreased Cr (VI). The outcomes of this study revealed that the isolate *A. flavus* had notable Cr (VI) reduction activity at 97%. The findings of Shankar *et al.* (2014), who found 86.7% activity of Cr (VI) reduction utilising *A. flavus*, are comparable to these findings. The elimination of Cr (VI) may also be accomplished by non-enzymatic organic acids and chromate reductase. The intracellular chromate reductase in *A. flavus* is primarily responsible for the reduction of Cr (VI), which may be converted inside the cell to the less hazardous Cr (III).

In this study, the enzyme's activity peaked at pH 7.0. The enzyme may be suitable for effective bioremediation of Cr (VI) in the tannery effluent, given the activity shown at neutral pH. *Bacillus methylotrophicus* inducible chromate reductase with optimal pH activity also exhibits a comparable enzyme function (Sandanamala *et al.,* 2015). This is consistent with the results of other researchers who found that the optimal temperatures for activity for chromate reductase from *A. niger* and GST-ECNfsA (a cloned chromate reductase) were 50^{0}C and 55^{0}C, respectively (Kwak *et al.,* 2003; Sallau *et al.,* 2014)

In a related investigation, a number of fungi, including *A. niger, A. flavus, A. fumigatus, A. foetidus, and A. viridinutans*, were isolated from tannery effluent-polluted

soil samples and utilized to remove Cr (VI). At a pH of 3.0, a fungus biomass of 4 g, and an initial Cr (VI) concentration of 18.1 mg/L, *A. niger* (96.3%) outperformed *A. flavus, A. umigates, A. foetidus,* and *A. viridinutans* in terms of Cr (VI) reduction effectiveness. *A. flavus* was employed by Singh *et al.* (2016) to reduce Cr (VI) from simulated wastewater with the addition of Fe (II) ions. Many fungi, including *Rhizopus, Penicillium, Aspergillus niger*, and *Trichoderma*, are employed in the bioremediation of contaminated water (Dusengemungu *et al.*, 2020). Although bacteria need energy to digest the toxicants, fungi-based bioremediation techniques do not (Ismail and Moustafa, 2016). Partly examined is the mechanism underlying fungi's bioremediation of metals.

A protein expression profile revealed that the fungus cell had been exposed to chromium. When samples of tannery effluent are contaminated with Cr (VI), their source location may be the reason for this Cr (VI) tolerance potential (Narayanan *et al*., 2021). This is consistent with the results of other researchers who found that the optimum temperatures for chromate reductase from *A. niger* and GST-ECNfsA (a cloned chromate reductase, respectively) were similar and close at 50°C and 55°C (Kwak *et al.,* 2003; Sallau *et al.,* 2014). The ability of fungi to withstand metals at high concentrations allows them to thrive in hostile environments like Cr (VI) and other metal stress situations.

However, the low K_M value found for the chromate reductase isolated from *A. flavus* indicates that the enzyme has a high affinity for the substrate chromate. Additionally, this value is lower than the K_M found in the reports made by other researchers for chromate reductases isolated from various microbial sources (Sallau *et al.,* 2014; Mohammed *et al*., 2021). The enzyme has a higher maximum velocity (Vmax) than chromate reductase isolated from a related fungus, *A. niger* as reported by Sallau *et al.,* 2014. As greater Vmax values have been reported for chromate reductases from other bacterial sources, this implies and also place $Cr^{6}+$ to $Cr^{3}+$ appropriately than chromate reductase from *A. niger* (Park *et al.,* 2000; Mohammed *et al.,* 2021).

CONCLUSION

The findings of this study revealed that A. flavus, which is a chromium-resistant fungus, is capable of reducing or transforming Cr (VI) to less toxic Cr (III). Consequently, it reduced a significant percentage of Cr (VI) in 3 days of bioremediation. The results implied that the fungal strain A. flavus can be used for the remediation of Cr (VI) in tannery effluent-contaminated sites. Bioremediation using indigenous fungal strains to remove Cr (VI) from contaminated sites can be regarded as an alternative to other known techniques because of its short action period and better accumulation efficiency.

AUTHORS' CONTRIBUTIONS

SYM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Data curation. SIRO: Design of the study, Supervision, Writing – review & editing. MB: Formal analysis, Resources. ISI: Formal analysis, MSS: Project administration, Supervision. BEI: Writing – original draft, review & editing. EIA: Validation; Visualization. All authors approved the revised final version and consent for its publication.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose

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