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Short communication

Analyses of carcinogenic aromatic amines released from harmful azo colorants by *Streptomyces* SP. SS07

Malayappan Bhaskar[a], Arumugam Gnanamani[b], Rama Jeevan Ganeshjeevan[a], Raghavan Chandrasekar[a], Sayeed Sadulla[b], Ganga Radhakrishnan[a,*]

[a] *Eco Analytical Laboratory, Central Leather Research Institute, Adyar, Chennai 600020, Tamil Nadu, India*
[b] *CHORD Division, Central Leather Research Institute, Adyar, Chennai 600020, Tamil Nadu, India*

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Abstract

Extracellular fluid protein (ECFP) of *Streptomyces* species SS07 has been used to reduce water soluble azo dyes and the carcinogenic amines released have been compared with that from chemical reduction. The effect of temperature, pH and contact time on the recovery of amines using ECFP was studied. The ECFP releases carcinogenic amines at a pH of 9.2 and a temperature of 37 °C for a contact period of 24 h. The reduction products were analyzed with HPLC and their structures confirmed by LC–MS and GC–MS. It was observed that both the ECFP and chemical reduction methods released similar type of amine products. In the case of dye samples, compared to chemical reduction, 5–20% increase in the release of carcinogenic amines by ECFP was observed. The percentage of amine products released by chemical reduction was higher for leather garment samples compared to ECFP treatment.
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1. Introduction

Azo dyes are important colorants having extensive application in textiles, papers, leathers, gasoline, additives, foodstuffs and cosmetics [1]. It is well known that soluble azo dyes when incorporated into the body are split into corresponding aromatic amines by liver enzymes and intestinal micro flora [2,3]. Several aromatic amines including benzidine, 4-aminobiphenyl and 2-napthylamine have been classified by the International Agency for Research on Cancer (IARC) as known human carcinogens [4–6]. These aromatic amines pose health hazards to human beings in two ways viz. direct contact and through environment. The release of aromatic amines from consumer products such as clothing and leather has already been reported [7,8]. Hence, it is necessary to ensure that our clothing, footwear and other personal wear are free from carcinogenic amines. Though the dye molecule is biologically inactive, microorganisms in skin or in

* Corresponding author. Tel.: +91-44-24430268; fax: +91-44-24430267.
E-mail address: clrieco@md3.vsnl.net.in (G. Radhakrishnan).

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environment can cleave the azo linkages and release the respective amines [9–11], and the subsequent absorption of aromatic amines *via*. skin by skin bacteria has been reported [12]. The role of *Streptomyces* sp. on the decolorization of azo dyes, Poly B-411, Poly R-418 and brilliant blue have been reported by several research groups [13–15]. Hence, the role of microbes in reducing azo dyes to toxic carcinogenic compounds is of considerable concern [16]. In view of these findings, some member states of the European Union (EU) have introduced a ban on azo colorants used in consumer goods, which might release any of the 20 listed harmful aromatic amines. At present, the German method DIN 53316 is the most accepted analytical method for the determination of azo colorants in leather and dyes [17].

The present study has been taken up to (i) evaluate the quantity of amines released by the extracellular fluid protein (ECFP) of *Streptomyces* sp. and (ii) to compare the percentage recovery with chemical reduction. Influence of temperature, ECFP concentration and contact time on the release of amine compounds from dyes samples have also been evaluated. The release of amines from leather garment samples treated with ECFP was studied in both acidic and alkaline sweat medium. Instrumental techniques such as HPLC, UV-Vis spectrophotometry, LC–MS and GC–MS have been adopted to quantify and characterize the products released.

2. Experimental

2.1. Materials

The dye sample xylidine ponceau-2R (XP-2R) has been procured from Sigma Chemical Co. (St. Louis, MO, USA). Direct black-38 (DB-38) and direct brown-1 (DB-1) were obtained from commercial sources. Stock standard solutions of amines were prepared in methanol at a concentration of 1000 μg/ml. The standard solutions were then diluted with methanol to appropriate concentration. All the solutions were stored in the dark at 4 °C. Sodium dithionite solution (0.2 g/ml) used for the chemical reduction was prepared in double distilled water. Fresh solutions were used always. HPLC analysis grade water was prepared with ELGASTAT maxima HPLC of ELGA, Bucks, UK.

2.2. Analyses conditions

A UV-Vis spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) lambda 14 model driven by UV-Win software version 1.4 was used for the entire study.

HPLC separation was carried out using Waters Alliance 2695 separation module with a high-pressure gradient pump, Alliance autosampler and a photodiode array detector (PDA 996, Waters Milford, MA, USA). The amines were separated on a purosphere RP-18 column (5 μm), Merck KA, Darmstadt, Germany. The mobile phase consisted of acetonitrile (eluent A) and water (eluent B). Separations were accomplished at a flow rate of 0.7 ml/min, at 25 °C and injection volume 10 μl, at wavelengths of 240 and 280 nm. Millennium 3.2, Waters chromatographic data system software was used for data processing. Confirmation of the product was made with the chromatogram and the corresponding UV spectra of the standard amines. Mass spectrum analyses were performed using API-150 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) operated using electron spray ionization with ion spray source operated in positive ion mode. Instrument control and data acquisitions were performed with Macintosh G4/400 computer (Apple, Cupertino, CA, USA) using Mass Chrom 1.1.1 software (PE Sciex). The nebuliser gas flow and the curtain gas flow (N_2) were set at 10 and 8 l/h, respectively. The ion spray, orifice and ring voltage were set at 4800, 40, 170 V respectively. The structure of the amines, benzidine and 4-aminobiphenyl were confirmed by LC–MS, whereas xylidines and trimethylaniline, which exhibited poor detectability in LC–MS were analyzed by GC–MS. GC–MS model 800 with VG Mass Lab software version 1.3 (Fisons Instruments, Milan, Italy) was used for analyzing xylidines and trimethylaniline. DB-5 MS mid polar column of 30 m × 0.32 mm i.d. (film thickness: 0.25 μm) from J & W Scientific (Folsom, CA, USA) was used. GC condition was set as: injection in splitless mode; injection temperature: 250 °C; oven temperature: 50–280 °C; ramped at 5 °C; and interface temperature: 250 °C. Mass detection mode was electron impact ionization (EI) positive, set at 70 eV, scanned from 50 to 800 amu with total ion

current mode of detection. Helium was used as carrier gas at 1.5 ml mass flow rate. One microliter of the sample in methanol was injected.

2.3. Isolation of Streptomyces sp.

Streptomyces sp. was isolated from the soil, which was in contact with wastewater from food industry as per the method summarized in [18]. Identification of the isolate was made based on morphological and biochemical examination according to William et al. [19]. The basophilic nature of the isolated species is an added feature of this organism and hence used for the present study. The optimum pH for maximum growth was 9.2–9.5 and the optimum temperature was 37 °C. Pure culture was obtained upon sequential screening and the stock cultures were maintained at 4 °C. Periodical subcultures were made at 30 days interval. The protein content of the extra cellular fluid protein was estimated according to the method of Lowry et al. [20].

2.4. Release of amines by chemical method

Dye samples (0.1 g) were treated with sodium dithionite (three times with 1.5 ml of 0.2 g/ml solution) in an aqueous sodium citrate buffer solution (pH 6.0) at 70 °C for 30 min in a closed vessel. The amines released in the process of reductive cleavage were extracted with 50 ml of chloroform [21] and finally concentrated to 2 ml. The remaining solvent was removed by flushing nitrogen. The residue was then dissolved in methanol and analyzed by HPLC–diode array detection (DAD).

2.5. Release of amines by extra cellular fluid protein (ECFP)

About 0.1 g of dye samples was treated with ECFP, dissolved in water and incubated at 37 °C. The carcinogenic amines released were analyzed by UV-Vis, HPLC, LC–MS and GC–MS. Dye samples with ECFP were incubated for 1 and 24 h period at 37 °C to assess the optimum contact time between ECFP and the dye sample for the release of amines. The optimum concentration of ECFP required for complete degradation of azo dye samples was determined by varying the ECFP concentration from 0.785 to 3.925 mg of protein/mg of dye. All experiments were duplicated.

2.6. Release of amine compounds from leather garment sample by chemical and ECFP treatment

Leather garment sample dyed with DB-38 or DB-1 was chosen for the study. The leather samples were ground using a laboratory mill (Thomas Wiley model 4, Thomas Scientific, USA). For chemical reduction, about 1.0 g of the ground leather sample was treated with hexane to remove oils and fats [17] and reduction was carried out in acidic and alkaline sweat medium with sodium dithionite. The analyses of the reduction products are as given in Section 2.4. For enzymatic reduction, the sample was washed with water and then the wet residue was treated with ECFP. Influence of ECFP on the release of aromatic amines from leather garment samples was assessed in both acid and alkaline sweat conditions [22]. The incubation period and analyses of the products are as per the procedure in Section 2.5.

3. Results and discussion

3.1. UV and HPLC analyses

The λ_{max} values of raw dyes and products of ECFP treatment are given in Table 1. UV-Vis analysis of the samples with ECFP showed no peak corresponding to the λ_{max} of the raw dyes. The results clearly show the complete reduction of the azo dyes to the corresponding aromatic amines. HPLC analyses revealed that both chemical and enzymatic reduction released similar aromatic amines and the results are compiled in Table 1. It was observed that the carcinogenic amines released by the ECFP treatment were 5–20% higher than those obtained in chemical reduction. Representative HPLC chromatograms for enzymatic reduction are given in Fig. 1. The structure of dyes and their reduction products are given in Fig. 2.

3.2. LC–MS/GC–MS studies

The reduction of azo compounds to the corresponding aromatic compounds depends on the electron

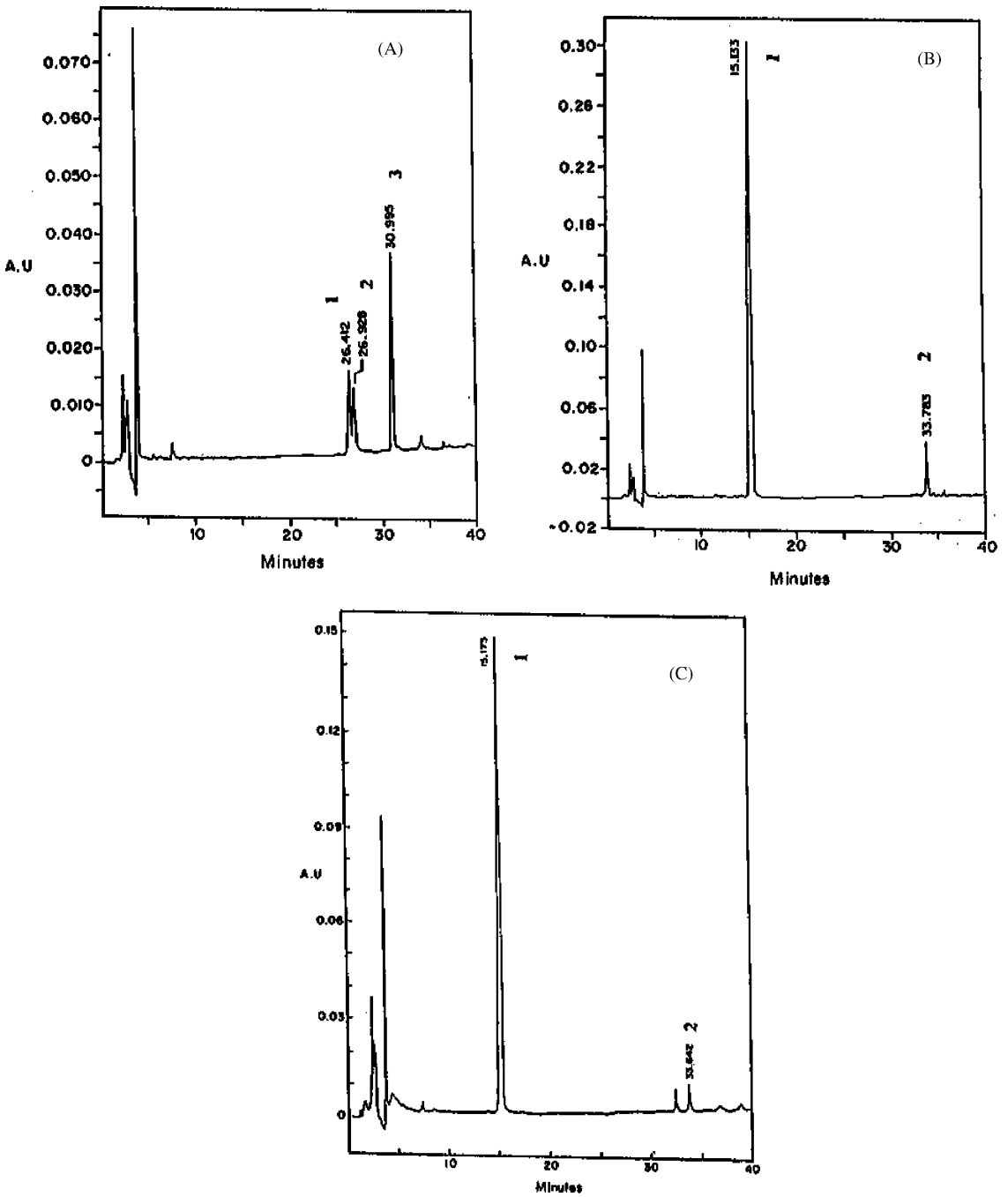

Fig. 1. HPLC chromatograms for enzymatic reduction. (A) Xylidine ponceau-2R (1 = 2,4-xylidine, 2 = 2,6-xylidine, 3 = 2,4,5-trimethylaniline). (B) Direct black-38 (1 = benzidine, 2 = 4-aminobiphenyl). (C) Direct brown-1 (1 = benzidine, 2 = 4-aminobiphenyl). Conditions: mobile phase, acetonitrile (A) and water (B); flow rate, 0.7 ml/min; 25 °C; injection volume, 10 μl; gradient elution: 0 min, A 23%, B 77%; 0–21 min, A 34% and B 66%; 21–30 min, A 60% and B 40%; 30–34 min, A 70% and B 30%; 34–37 min, A 90% and B 10%; and 37–40 min, A 23% and B 77%. Detection at 280 nm.

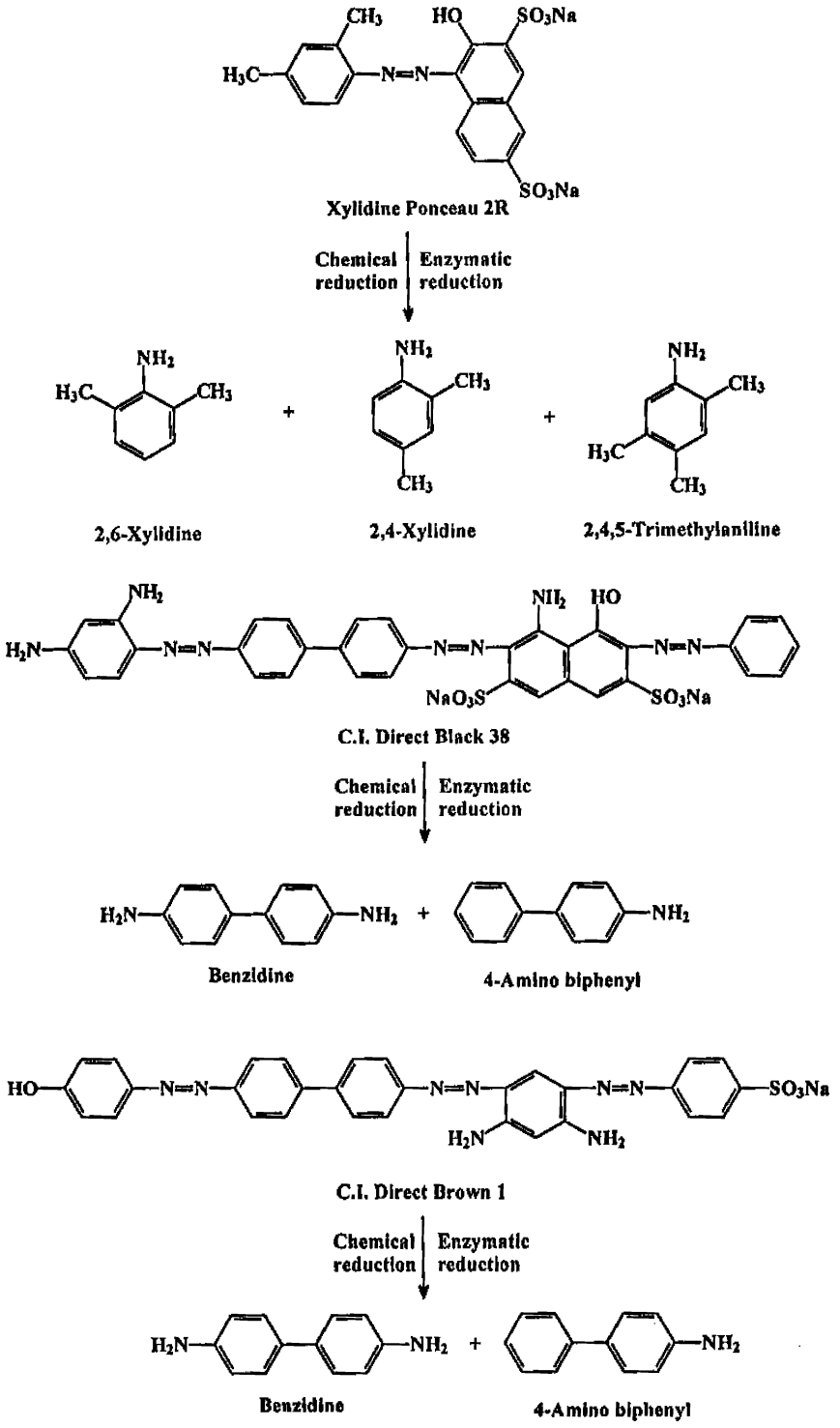

Fig. 2. The structure of dyes and their reduction products.

Table 1
UV-Vis, mass spectral and HPLC data for recovery of amines in both chemical and enzymatic reduction

Serial number	Dyes	λ_{max} (nm)		Amines	Mass spectral data (m/z)		Retention time (t_r) (min)	Recovery of amines (ppm)	
		Raw dye	After ECFP treatment		Theoretical	Observed		Enzymatic reduction	Chemical reduction
1	Xylidine ponceau-2R	506.15	287.52	2,4-Xylidine	121	121[a]	26.412	42,680	46,600
				2,6-Xylidine	121	121[a]	26.928	18,090	19,190
				2,4,5-Trimethyl-aniline	135	135[a]	30.995	65,330	50,400
2	Direct black-38	597.77	287.51	Benzidine	184	185 $(M + H)^{+}$[b]	15.133	31,870	24,680
				4-Aminobiphenyl	168	169$(M + H)^{+}$[b]	33.783	3,830	1,770
3	Direct brown-1	520.67	287.15	Benzidine	184	185 $(M + H)^{+}$[b]	15.173	35,770	25,550
				4-Aminobiphenyl	168	169 $(M + H)^{+}$[b]	33.642	1,130	3,590

[a] Analyses done by GC–MS.
[b] Analyses done by LC–MS.

density around the –N=N– groups. Initial decolorization of azo samples by the bacterial and fungal species during contact with the dyes may be due to the removal of chromophoric groups [23]. LC–MS and GC–MS analysis of the treated samples provide evidence for the presence of stable amine products in the aqueous phase. LC–MS (ESI mode) analyses of the aromatic amines showed that the reduction products of DB-38 and DB-1 were benzidine and 4-aminobiphenyl which gave the fragmentation pattern with the formation of $[M + H]^{+}$ as the base peaks at $m/z = 185$ for benzidine and $m/z = 169$ for 4-aminobiphenyl. XP-2R released xylidines and trimethyl aniline, which showed poor sensitivity in LC–MS, and they were analyzed by GC–MS which showed $m/z = 121$ for 2,4-xylidine and 2,6-xylidine and $m/z = 135$ for 2,4,5-trimethylaniline. To confirm that the response is only from the corresponding amines and not from any other organics, UV spectra of the analytes with the reference compounds were analyzed under similar conditions. Samples showed the same chromatogram and the UV spectrum with identical mass spectra. The samples were quantified with the area of the standard under the same experimental conditions. The Table 1 gives the mass spectra of the corresponding amines.

3.3. Effect of contact time

On treatment with ECFP, all the three dyes release products similar to the products obtained through chemical reduction. Regarding the contact time, i.e. the incubation period, high recovery was observed after 24 h. After 24 h contact time, about 75% of 2,4-xylidine, 32% of 2,6-xylidine, and 70% of 2,4,5-trimethyl aniline were recovered from XP-2R. From DB-38, about 75% of benzidine and 5% of 4-ABP and from DB-1, 70% of benzidine and 13% of 4-ABP were recovered.

Table 2
Percentage release of amines from leather garment sample

Serial number	Dye	Enzymatic reduction (%)				Chemical reduction (%)			
		Acidic sweat		Alkaline sweat		Acidic sweat		Alkaline sweat	
		Benzidine	4-Amino biphenyl	Benzidine	4-Amino biphenyl	Benzidine	4-Amino biphenyl	Benzidine	4-Amino biphenyl
1	C.I. direct black-38	25.10	12.70	57.74	28.05	70.55	31.20	85.77	42.64
2	C.I. direct brown-1	28.60	14.98	54.92	32.84	61.23	27.54	79.65	43.57

3.4. Release of amines from dye samples with varying ECFP concentration

To evaluate the quantity of ECF protein required for maximum recovery of amines, concentration of ECFP was varied from 0.785 to 3.925 mg/mg of dye sample. The optimized concentration of ECFP for maximum recovery was 3.14 mg/mg of dye sample and the maximum percentage of amines recovered are given in Section 3.3. Further increase in protein concentration does not effect any further reduction.

3.5. Release of amine products from leather garment sample

The percentage of amines released from leather garment samples by ECFP and chemical reduction under different experimental conditions (acidic and alkaline sweat medium) are given in Table 2. It is interesting to observe that the percentage release of amine compounds by chemical method is higher than the enzymatic treatment. More than 40% of benzidine and 4-aminobiphenyl were released under chemical reduction and it was <20% under enzymatic reduction. Also increased release of benzidine and 4-ABP was observed in alkaline sweat medium compared to acidic sweat medium. The presence of hydroxyl ion in the aqueous phase enhances the cleavage compared to hydrogen ion, which is attributed to the formation of higher percentage of amine products from leather garment sample in alkaline medium.

4. Conclusion

In the present study, reduction of azo dyes to corresponding aromatic amines by extra cellular fluid protein isolated from streptomyces species and a comparison with the dithionite reduction method has been made. Although both chemical and enzymatic reduction release similar amines, enzymatic reduction yielded higher percentage of major and minor amines. Structure of the released amines were confirmed by GC–MS and LC–MS techniques. In the case of leather garment samples, the percentage of amines released was higher with chemical reduction as compared to enzymatic reduction. Our study proves that the ECFP produced by *Streptomyces* sp. plays a key role in releasing carcinogenic amines.

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