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Screening and Optimization of Environmental Parameters for Maximum Decolorization of Reactive Orange 122 Azo- dye by *Streptomyces* sp (A5)

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ABSTRACT

The aim of current study was to test the ability and optimize the decolorization of textile azo Reactive Orange 122 by *Streptomyces* sp (A5) isolated from dye contaminated soil. The decolorization ability of the isolate *Streptomyces* sp (A5) was assessed by spectrophotometer at 3, 5 and 7 days in starch nitrate broth media amended with 0.35 g/L of the Reactive Orange 122 azo dye. Different incubation conditions like concentration of the dye, temperature and pH were used in the present study to investigate their effect on the decolorization rate. The potential isolate *Streptomyces* sp (A5) exhibited significant decolorization ability at 5 days of incubation. The optimum conditions were found for degradation of Reactive Orange 122 azo dye by the isolate *Streptomyces* sp (A5). The maximum degradations were noticed at 0.3 g/L of dye concentration, 6 pH and 35 °C temperature, respectively. In conclusion, the results of the current study showed that the isolate *Streptomyces* sp (A5) was effectively degraded the textile dye Reactive Orange 122 azo dye under the optimized conditions.

INTRODUCTION

Azo dyes, now are considered the largest group of the used synthetic dyes (Chung 2016; Imron *et al.*, 2019). Due to its large usage in different industries including plastic, textiles, the printing of both paper and leather, Azo dyes can lead to some wastes in the environment (Chung 2016; Sarkar *et al.*, 2017), which in turn causes serious effects to the environment and its ecosystems, it can damage the aquatic ecosystem through water pollution leading to a reduction in light penetration to organisms living in water which finally, leads to a disruption photosynthesis process (Al Farraj *et al.*, 2019). Due to its toxicity and carcinogenicity to human and other living organisms (Khataee *et al.*, 2010).

Therefore, many literatures has recognized the importance of Azo dye removal and degradation from the environment especially from water as an important source for all living organisms (Adnan *et al.,* 2014; Garg *et al.,* 2004; Hadibarata and Nor, 2014; Lai *et al.*, 2017; Tan *et al.* 2016). The solutions for this big problem can be categorized into three groups, which largely used to remove Azo dyes from the environment, these technologies are name chemical, physical and biological ones (Al Farraj *et al.,* 2019; Hadibarata *et al.,* 2018; El Hassani *et al.*, 2019). Due to the drawbacks of both chemical and physical methods for treatment which include low efficiency, living high amounts of sludge, high energy consumption and high costs (Azimi *et al.*, 2017). Therefore, the philosophy of decolorization need to be improved, biological methods seem to be the ideal methods for the removal of azo dyes pollution because it is eco-friendly, produce almost no other toxic wastes or sludge in the environment and finally, it's a low-cost treatment in comparison with physical and chemical one (Bilal *et al.,* 2019; Al Farraj *et al.,* 2019). The biological method includes using microorganisms to remove the pollutants through their metabolic pathways (Imron *et al*., 2019b). Recently, a large number of research studies using both macroorganisms as plants and microorganisms in wastewater treatment for removing of azo dyes, among these organisms bacteria (Ayed *et al.,* 2017); algae (El-Sheekh *et al*., 2009) ; Actinomycetes (Buntić et al. 2017); fungi (Fu and Viraraghavan, 2002; Shedbalkar e*t al.,* 2008); yeast (Jadhav *et al.*, 2007) and higher plants (Imron et al., 2019a; Singh and Singh, 2017). Considering the aforementioned huge research necessity, the aim of present study was to evaluate the biodegradation ability of *Streptomyces* sp (A5) towards Reactive Orange 122 azo. Moreover, the effect of the initial concentration of dye, pH and incubation temperature was also investigated. Finally, our findings during the present work can be a useful tool for microbiologists concerning with the environmental issues in designing new environmental strategies using microorganisms.

MATERIALS AND METHODS

1. Collection of Samples:

Different types of samples were collected. Firstly, soil samples were collected in polyethylene bags during 2019 from agriculture soils from three different localities in Sharqia (30.7°N 31.63°E), Dakahlia (31.3°N 31.23°E), Aswan (24.08°N 32.89°E) and Monufia (30.52°N 30.99°E). Secondly, water samples (seawater) were collected from Alexandria (31.12°N 29.55°E), and South Sinai (Dahab city) (28.29°N 34.30°E). All the collected samples were transferred to the laboratory of microbiology at Faculty of Science, Benha University and kept for further processes.

2. Isolation of Streptomycetes:

streptomycetes were isolated according to the method of Hayakawa and Nonomura (1987). Briefly, 10 g of soil sample / 10 ml of water sample were transferred into 90 mL of sterilized NaCl solution (0.85% w/v) in Erlenmeyer conical flasks, shaken for 15 min, one mL of the supernatant was inoculated into Starch nitrate agar (10 g Starch, 2 g KNO_3, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g NaCl, 2 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 20 g Agar and 1 L seawater) (Waksman, 1961). To eliminate moisture films on the agar plate, Petri dishes were prepared one day before inoculation and incubated at 37° C overnight (Shearer, 1987). 0.1 mL of the prepared suspension with proper dilution was placed on each plate and spread through a sterile glass rod. For water samples, different concentrations of seawater (25%, 50%, 75% and 100%) were inoculated on the same previous isolation media. Then plates were incubated at 28°c and after 2-4 weeks the bacterial growth was examined to allow the development of slow growing organisms. Based on their morphological characters as deep sitting colonies, sporulation and colors Streptomycetes were selected and purified on starch nitrate agar

and kept for further uses. Isolates were serially numbered with letters according to the sample site with a serial numbered e.g., Monufia (M), Aswan (A), Sharqia (S), Dakahlia (D), Alexandria (Alex) and South Sinai (Dahab city) (DC).

3. The General Condition of Testing Methods:

For diagnostic tests, all the reagents and media were prepared from analytical-grade chemicals. Sterilization of media was made at 121°C for 15 minutes. One week old slant or broth culture was used for inoculations of test media and the incubation of diagnostic tests was carried out at 28°C. All tests and experiments were made in duplicates. Strains were routinely maintained on starch nitrate agar.

4. Qualitative Assay of Azo dye Bio-degradation:

Isolated Actinomycetes was inoculated on starch nitrate agar media (Waksman, 1961) modified through adding 0.35 g/L Mono Azo dye (Reactive Orange 122) (**Figure 1**), and the Bio-degradation was observed with the naked eye as a positive or negative result by forming a clear zone around the growing colonies.

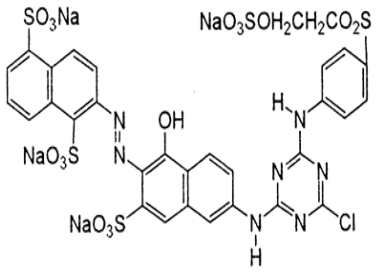

Fig.1. Chemical structure of Reactive Orange 122

5. Identification of Selected Isolate:

The most potent isolates capable of decolorization of azo dye were identified depending on several aspects such as morphological characters and biochemical tests as described in Bergey's manual of determinative bacteriology (Bergey *et al.*, 1939; Szabó *et al.*, 1975).

6. Quantitative Assay of Azo dye Bio-degradation:

Streptomycetes isolates which showed the best Bio-degradation ability was inoculated on modified broth starch nitrate media amended with 0.35 g/L Mono Azo dye (Reactive Orange 122). Briefly, 50 mL of media was inoculated by 1 mL of broth culture and incubated in a shaker 150 rpm at 28°C. Then 3 mL of culture medium was withdrawn after 3, 5, and 7 days and centrifuged at 10,000 rpm for 15 min to separate the cell mass. Decolorization of the dyes was determined by measuring the absorbance of the decolorization medium at 500 nm (Kalyani et al., 2008). Finally, the percentage decolorization was calculated as follows (Al Farraj *et al.*, 2019):

$$\text{Decolorization (\%)} = \frac{A-B}{A} \times 100$$

A: Initial absorbance

B: Observed absorbance

7. Optimization of Environmental Parameters That Affect the Decolorization Process:

Various factors were optimized to achieve the highest decolorization rate of different azo dyes using the selected potent *Streptomyces*. All the experiments were conducted in triplicate. A decolorization experiment was performed for all the following parameters as described before in section 6.

7.1. Effect of Different Incubation Temperature:

Biodegradation activity on Mono Azo dye (Reactive Orange 122), by *Streptomyces*

sp was studied at different incubation temperatures such as 25, 30, 35 and 40˚C.

7. 2. Effect of Different Incubation PH Value:

Biodegradation activity on Mono Azo dye (Reactive Orange 122), by *Streptomyces* sp was studied by incubation at different pH values of the medium ranging from 5 to 9 (1 interval), the pH was adjusted by using 0.1N HCl and 0.1N NaOH solutions.

7. 3. Effect of Dye Concentration on Biodegradation:

Biodegradation activity on Mono Azo dye (Reactive Orange 122), by *Streptomyces* sp was studied by inoculation the isolate on starch nitrate broth amended with different concentrations (0.2 g/L, 0.3 g/L and 0.4 g/L) of Reactive Orange 122 azo dye.

8. Statistical analyses

All the experiments were carried in triplicate and the results were tabulated as mean ± STDEV and the data was analyzed using GraphPad Prism, www.graphpad.com"

RESULTS

1. Isolation and Screening of Azo Dye Degrading Streptomyces Fungi:

Randomly (101) streptomycetes isolates were isolated from different environments including soils and water (seawater) on starch nitrate agar media. Streptomycetes isolates were selected according to the special morphology of the colony which has rounded and convex shape colonies, with deeply rooting growth into the medium. Colonies are usually covered with dry and powdery spore masses. Monufia gives the highest number of recovered isolates followed by Aswan, Sharqia and Dakahlia, while the lowest number from Alexandria and South Sinai (Dahab city) (**Table 1**).

Table 1. Total number of streptomycetes isolates.

	Locality	No. of isolates	Incidence percent (%)
soil	Monufia	43	41.74
	Dakahlia	12	11.65
	Sharqia	15	14.56
	Aswan	25	24.27
Marine	Alexandria	5	4.86
	South Sinai (Dahab city)	3	2.92
	Total	103	100

2. Qualitative Assay of Reactive Orange 122 Azo dye Bio-degradation:

The ability of isolates on Azo dye Bio-degradation was assessed through culturing on starch nitrate medium amended with Azo dye (Reactive Orange 122). Some isolates showed good results and the others did not show any degradation (**Table 2**). The table shows that isolate (A5) was the best isolate which gave high degradation. This isolate was selected for further studies

Table 2. Qualitative assay of Azo dye (Reactive Orange 122) Bio-degradation

Monufia's isolates							
Isolate No.	**Result**	**Isolate No.**	**Result**	**Isolate No.**	**Result**	**Isolate No.**	**Result**
M1	-	M12	-	M23	-	M34	++
M2	-	M13	-	M24	+	M35	-
M3	-	M14	-	M25	-	M36	-
M4	++	M15	++	M26	++	M37	++
M5	-	M16	-	M27	-	M38	-
M6	-	M17	+	M28	-	M39	-
M7	-	M18	-	M29	-	M40	+
M8	+	M19	-	M30	-	M41	-
M9	-	M20	+	M31	+	M42	-
M10	-	M21	-	M32	-	M43	-
M11	-	M22	-	M33	-		
Dakahlia's isolates							
Isolate No.	Result	Isolate No.	Result	Isolate No.	Result	Isolate No.	Result
D1	-	D4	-	D7	-	D10	++
D2	-	D5	-	D8	-	D11	-
D3	+	D6	-	D9	-	D12	-
Sharqia's isolates							
Isolate No.	Result	Isolate No.	Result	Isolate No.	Result	Isolate No.	Result
S1	-	S5	+	S9	-	S13	-
S2	-	S6	-	S10	+	S14	-
S3	++	S7	-	S11	-	S15	++
S4	-	S8	-	S12	++		
Aswan's isolates							
Isolate No.	Result	Isolate No.	Result	Isolate No.	Result	Isolate No.	Result
A1	++	A7	++	A13	+	A19	++
A2	++	A8	-	A14	++	A20	++
A3	-	A9	+	A15	+	A21	-
A4	++	A10	+	A16	-	A22	-
A5	+++	A11	-	A17	-	A23	-
A6	+	A12	+++	A18	++	A24	-
Alexandria's isolates							
Isolate No.	Result	Isolate No.	Result	Isolate No.	Result	Isolate No.	Result
Alex1	++	Alex2	-	Alex3	-	Alex4	++
South Sinai (Dahab city) isolates							
Isolate No.	Result	Isolate No.	Result	Isolate No.	Result	Isolate No.	Result
DC1	-	DC2	+	DC3	-		

(-) = negative (+++) = high degradation
(++) = medium degradation (+) = low degradation

3. Identification of Selected Isolate:

The morphological features of the potent isolate capable of decolorization of azo dye were observed on different growth media and identified according to Bergey's manual of determinative bacteriology. The isolate was provisionally identified up to the genus as *Streptomyces* sp (A5) (**Table 3)**.

Table 3. Morphological, physiological and biochemical characteristics of A 5 isolate

Morphological and cultural characteristics								
Spore chain morphology		Spore surface ornamentation		Color of spore mass		Pigmentation of substrate mycelium		Diffusible pigment
Straight/Flexuous>40		Smooth		gray		gray		-
Physiological and biochemical characteristics								
Melanin pigment production		Degradation activities				Nitrate reduction		H_2S production
peptone iron agar	tyrosine agar	xanthine	Elastine	Arbutin		+++		+
+	+	+	-	-				
Utilization of sugers								
D-fructose	Sucrose	Rhamnose	D-mannitol	D-xylose	Raffinose	I-inositol	Galactose	L-arabinose
-	+	+	-	+	+	-	+	+

4. Quantitative Assay of Reactive Orange 122 Azo dye Bio-degradation:

The Biodegradation activity on Reactive Orange 122 Azo dyes by *Streptomyces* sp (A5) at different incubation times. The data presented in **Figure 2** shows that the Biodegradation after 5 days was 51.12% which was significantly higher than that after 3 days 22.13%. While there was no significant increase after 7 days than the fifth day.

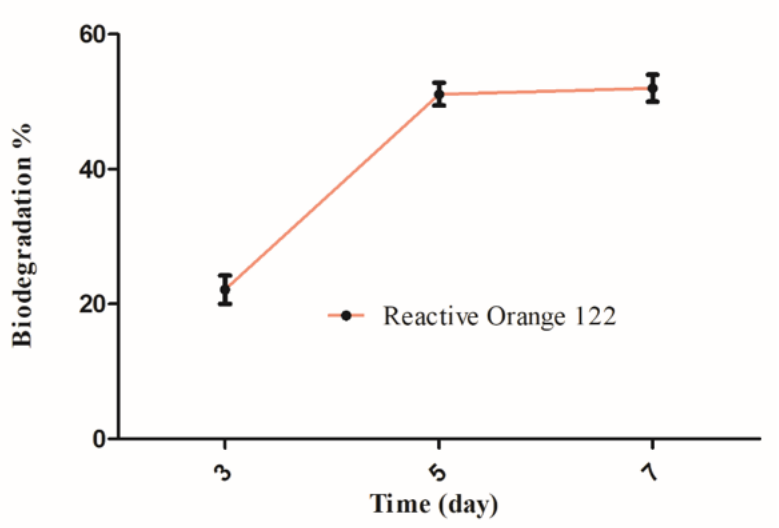

Fig. 2. Biodegradation (%) of Reactive Orange 122 by *Streptomyces* sp A5 at different time.

5. Effect of Different Incubation Temperature:

The Biodegradation activity on Reactive Orange 122 azo dye by *Streptomyces* sp (A5) at different incubation temperatures was assessed. Data presented in **Figure 3**, shows that increasing temperature from 25°C to 35°C had a significant effect on increasing Azo dye Biodegradation and the optimum temperature was 35°C at which the Biodegradation was 62.47%. The Biodegradation rate was dropped with increasing temperature to 40°C and the Biodegradation was 44.61%.

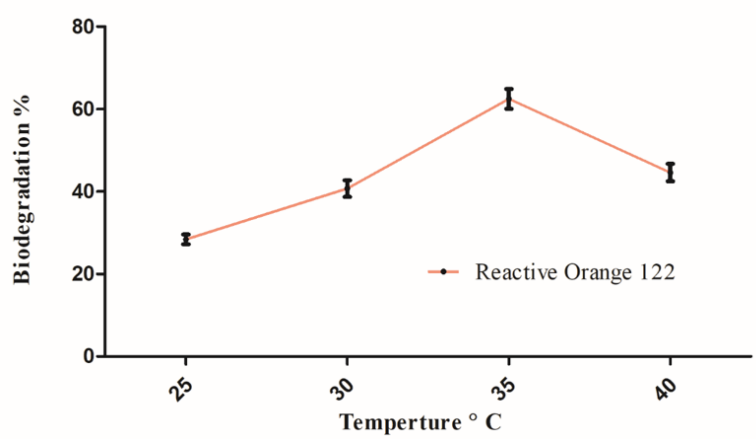

Fig. 3. Effect of Different incubation temperature on Biodegradation.

6. Effect of different inoculation PH value:

The Biodegradation activity on Reactive Orange 122 azo dye by *Streptomyces* sp (A5) at different inoculation PH was assessed. It was observed that in **Figure 4**, with increasing PH from 5 to 7 there is a significant increase in Azo dye Biodegradation and the optimum PH was 6 at which the Biodegradation was 69.1% which is significantly higher than PH 5. Also, Biodegradation rate was sharply dropped with increasing PH from 7 to 9 which is significantly lower than Biodegradation in the case of PH 6.

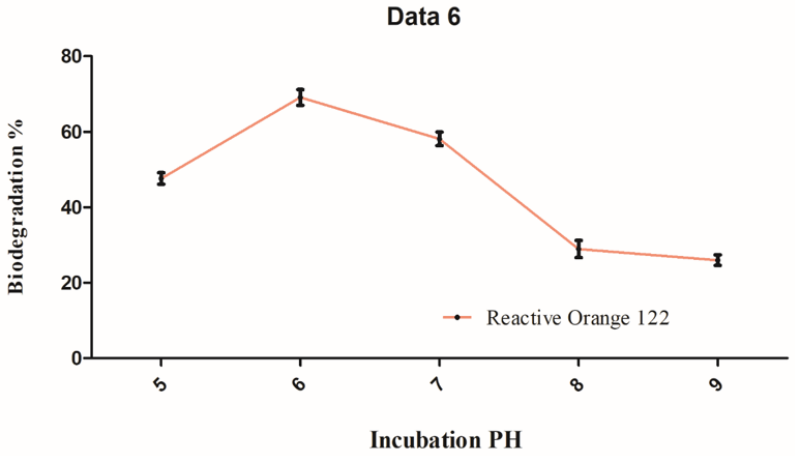

Fig.4. Effect of Different incubation PH on Biodegradation.

7. Effect of Dye concentration on Biodegradation.

The Biodegradation activity on Reactive Orange 122 azo dye by *Streptomyces* sp (A5) at different dye concentrations was assessed. It was observed that in **Figure 5**, with increasing dye concentration from 0.2 g/L to 0.3 g/L there is a significant increase in Azo dye Biodegradation and the optimum concentration was 0.3 g/L at which the Biodegradation was 60.74% which is not significantly higher than 0.4 g/L.

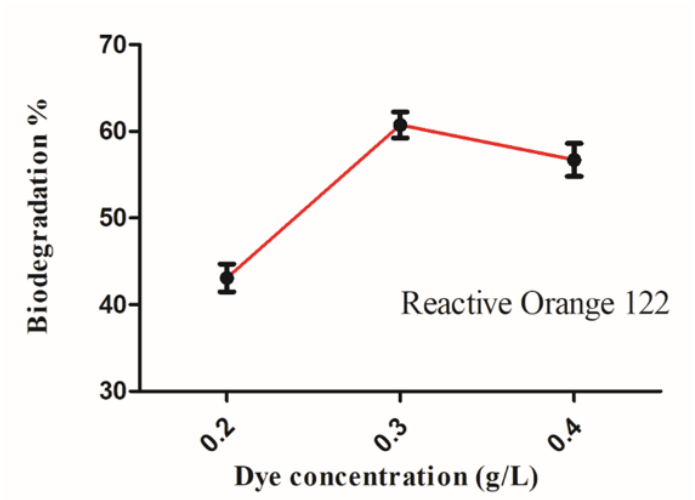

Fig. 5. Effect of Dye concentration on Biodegradation

DISCUSSION

Environmental pollution especially resulted from Azo dyes, which are the major class of commercially used dyes used in many industries as the textile industry had become a serious problem. Nearly, (3 X 10^5) tones of discharged textile dyes are added to the water environment worldwide (Ogugbue and Sawidis, 2011). These wastes had a bad impact not by changing the water character but also, by its carcinogenic capacity (Singh *et al.*, 2015a). Many physical and chemical treatment processes were had been used for decolorization and removal of these dyes wastes, but because of their high cost, new strategies must be used like biological methods which are low in cost and eco-friendly (Chen, 2006). Owing to the rising level of pollution in the environment, the microbial removal of toxic wastes became an important area of research. In the current study, *Streptomyces* isolates from the soil and marine environment were examined for their potential to degrade and decolorize azo dye Reactive Orange 122. A total of (101) *Streptomyces* isolates were obtained and the isolate (A5) showed the highest ability of dye decolorization. Similar studies showed that three isolates of marine Actinomycetes belongings to the genera of *Micromonospora* sp., *Streptomyces* sp. and *Micropolyspora* sp. Had a high ability to degrade and decolorize Amido Black (Raja *et al.*, 2016). Another study, *Nocardia* sp. reported was reported to have the ability to decolorize the Congo Red (Chakravarthy *et al.*, 2015). In the current study, isolate A5 showed a decolorization ability of 51.12% at a dye concentration of 0.35 g/L (Reactive Orange 122) within 5 days and it was identified to be *Streptomyces* sp. (A5). Previously, it was reported that *Nocardiopsis alba* isolated from dying wastewater can decolorize 85% of Reactive orange 16 at a concentration of 250 mg/L (Shobana and Thangam, 2012), and 85.6% for *Nocardiopsis* sp. *dassonvillei* (Chittal *et al.*, 2019). In another study, azo dye Reactive Red 5B (50 mg/L) was degraded by *Streptomyces* sp. VITDDK3 (Kannabiran et al., 2010). It was reported that some physical and chemical parameters as pH and temperature have a great effect on the degradation of many environmental (Saratale *et al.*, 2011; Singh *et al.*, 2015b). In the current study, the effect of varying inoculation pH, incubation temperature, and dye concentrations on the isolate ability for dye decolorization was studied. The highest decolorization was achieved by inoculation at a pH of 6. Earlier it was reported that *Streptomyces krainskii* SUK-5 decolorized Reactive Blue-59 completely at pH 8 (Mane *et al.*, 2008). Previous studies showed the important role of temperature in dye decolorization and degradation (Endo *et al.*, 2003; Isiguro *et al.*, 2005; Molina-guijarro *et al.*, 2009). In the current study highest decolorization (62.47%) was achieved at 35°C. in the same line, it was

reported that at 40°C the best decolorization was achieved by *Streptomyces* sp. (Endo *et al.*, 2003). While, for Reactive Orange 16 the isolate *N. alba* showed its highest decolorization ability 30°C (Shobana and Thangam, 2012). Dye concentration has a vital effect on degradation ability; dye decolorization gradually increases with increased dye concentration till critical concentration then gradually decreases again. Maximum decolorization (60.74%) was achieved at concentration (0.3 g/L). Isolate *Streptomyces* sp (A5) showed good decolorizing ability till a dye concentration of 0.3 g/L. these phenomena can be illustrated by the idea that the toxic effects of pure dye imposed on the microbial cells can result in low decolorization.

Conclusion

In conclusion, to solve dye removal problems several studies had been conducted. Marine and Soil environments have a large number of unexplored organisms capable of production of novel secondary metabolites. Hence in the current study, these environments were used as a source for isolating azo dye decolorizing *Streptomyces*. Isolate A5 identified as *Streptomyces* sp. (A5) gave a high decolorization ability of Reactive Orange 122 with 51.12% at a dye concentration of 0.35 g/L. The optimum pH, temperature, and dye concentration were investigated. Maximum decolorization occurred at pH 6, temperature 35°C, and 0.3 g/Ldye concentration. However, further research is required to better understanding and effective employment of these isolates.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data Availability

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

Ethics Statement

Not applicable.

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