IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 4 Ver. II (Jul - Aug. 2015), PP 88-95
www.iosrjournals.org

Biodecolourization of Textile Dyes by Effluent Adapted Bacteria

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Abstract: *The discharge of textile azo dyes to the environment is an issue of health concern and can harm especially the aquatic ecosystem. The use of microorganisms has been reported to be effective approach for remediation. Five bacterial isolates with the capability of decolourizing textile dyes were isolated from textile effluent and identified as Bacillus thuringiensis (Isolate A2, B6), Bacillus badius (Isolate B5, B9), Bacillus aneurinolyticus (Isolate C2) by different morphological, physiological and biochemical tests. Physicochemical parameters such as temperature, pH and inoculum concentration were optimized for the decolourization process. The optimum temperature, pH and inoculum size for the decolourization of three experimental dyes (Novacron Orange FN-R, Novacron Red FN-R, Terasil Green) were found 30 to 35 °C, 7.0 to 8.0, and 10% (v/v) respectively. The selected bacterial isolates showed different decolourization activities for three experimental dyes. The isolate B5 (Bacillus badius) decolourized 98% of initially added Terasil Green after 48 h of incubation at 35 °C in neutral pH. The present study suggests that the isolated Bacillus sp. can be utilized to treat reactive dyes containing waste water.*

Key words: *Bacillus sp., Decolourization, Physicochemical Parameters, Textile Dyes, Textile Effluent*

I. Introduction

Synthetic dyes are widely used in textile, paper, food, cosmetics and pharmaceutical industries [1, 2]. About 60 – 80% of these azo dyes consumed in textile processing are characterized by a typical double azo bond linkage (-N=N-), which is the most common chromophore of reactive dyes [3, 4]. The delivery of colour onto fabric is not an efficient process and up to 40% of the dyes are lost during the dyeing process [5-7]. Dyes are not easily biodegradable, because they are designed to remain stable and long-lasting colorants. Dye colours are visible in water at 1 mg L^{-1} concentration, whereas textile processing waste water, normally contain more than 10-200 mg L^{-1} dye, resulting in environmental problems [8]. Residual dyes in waste water and their breakdown products such as benzidine, naphthalene and other aromatic compounds are toxic, carcinogenic and mutagenic to living organisms [9-11]. Untreated dyes become persistent in the environment for a long period of time. For example, the half-life of hydrolysed Reactive Blue 19 is about 46 years at pH 7.0 and 25 °C [12]. The physicochemical methods e.g. filtration, specific coagulation and flocculation are effective but quite expensive and have many disadvantages and limitations [13, 14]. The treatment process involving microorganisms offers a cheaper and environmental friendly alternative for colour removal in textile effluents. The decolourization ability depends on the adaptability and the activity of selected microorganisms. There are several reports on bioremediation of textile azo dyes based on many microorganisms that are capable of degrading azo dyes, including bacteria, fungi, yeast, algae [15-23], but these studies are limiting. The present study deals with the isolation of bacteria from textile effluent, assessing their decolourization efficiency under laboratory condition and optimization of the factors influencing the process.

II. Materials And Methods

2.1 Sample collection

Textile effluent samples from Keya Dyeing and Knit composite Limited (KDKCL), Jaron Bazar, Konabari, Gazipur, Bangladesh were collected in sterile glass bottles and it was from eight different locations of discharge. Immediately after collection, each sample was placed in an insulated box with frozen refrigerant packs in an insulated box and transported to the Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh. The samples were preserved at 4 °C until further analysis.

2.2 Primary screening of dye decolourizing Isolates

The textile effluent samples were enriched by co-incubating in Nutrient Broth (HI-MEDIA, India) containing 100 mg L^{-1} Cibacron Red FN-R dye at 30 °C for 48 h. After that, 100 µL of enriched broth was spread on Nutrient Agar (HI-MEDIA, India) plate supplemented with 100 mg L^{-1} Cibacron Red FN-R dye and incubated at 30 °C for 48 h. After incubation, bacterial colonies showing clear zones were isolated as potential decolorizing bacteria as clear zones indicate the ability to degrade Cibacron Red FN-R [22]. Then following co-incubation of isolates with a dye namely Cibacron Red FN-R in Nutrient Broth (HI-MEDIA, India), the isolates were screened observing their ability to decolourize the dye as measured by a spectrophotometer (T60 PG-INSTRUMENTS, UK). These primarily screened organisms were stored at -80 °C (DF 8517, ILSHIN BIOBASE CO., LTD, Korea) by liquid freezing and coded as A2, B5, B6, B9 and C2.

2.3 Identification of primarily screened dye decolourizing isolates

The identification of selected experimental organisms (A2, B5, B6, B9,C2) were carried out by observing their cultural characters on Nutrient Agar, PEMBA (Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar) media, gram staining and para-sporal body staining properties, growth pattern in different temperatures, pH, NaCl concentrations and biochemical test results (Motility, Indole, Methyl Red, Voges-Proskauer, Citrate utilization, Catalase, Oxidase, Starch and Caseins hydrolysis, Lysine Iron Agar (LIA), Triple Sugar Iron (TSI) agar, Nitrate reduction, Gelatin liquefaction, Proteolysis, Urease and fermentation of Arabinose, Rhamnose, Trehalose, Melibiose, Glucose, Xylose, Sucrose, Mannitol, Arginine. All of these tests were done as suggested in Bergey's Manual of Determinative Bacteriology [24].

2.4 Media and commercial industrial dyes used in decolourization assay

A medium was developed for decolourization experiment. The composition of the medium (M) used in the present study was as follows: glucose (BDH, England): 8.0 g L^{-1}, yeast extract (OXOID, England): 0.34 g L^{-1}, NH_4Cl (MERCK, Germany): 0.84 g L^{-1}, KH_2PO_4 (MERCK, Germany): 0.134 g L^{-1}, K_2HPO_4 (BDH, England): 0.234 g L^{-1}, $MgCl_2.6H_2O$ (MERCK, Germany): 0.084 g L^{-1}, Nutrient Broth (HI-MEDIA, India): 2.0 g L^{-1}. Media pH was adjusted at 7.0 using 5 M sodium hydroxide (NaOH) and hydrochloric acid (HCl). The dyes used throughout the study were collected from 4H dying industry located at Karnafuly, Chittagong, Bangladesh. These are reactive azo dyes and frequently used in most of the textile industries in Bangladesh. Three textile dyes namely Novacron Orange FN-R, Novacron Red FN-R, Terasil Green were used in the present study.

2.5 Dye decolourization at different parameters

The medium M was sterilized and supplemented with 100 mg L^{-1} filter sterilized experimental dyes (Novacron Orange FN-R, Novacron Red FN-R, Terasil Green). For the observation of temperature effect 10 ml dye supplemented media were inoculated with 10% (v/v) inoculum of experimental isolates (A2, B5, B6, B9, C2) and incubated at 20 °C, 30 °C, 35 °C, 40 °C temperatures. To observe the pH effect, 100 mg L^{-1} dye supplemented media prepared with different pH (5, 6, 7, 8, 9) were inoculated with the isolates and incubated at 30° C and to determine the effect of inoculum concentration 100 mg L^{-1} dye containing media were inoculated with 5%, 10%, 15% and 20% (v/v) bacterial suspension and incubated at 30 °C for 48 h. The decolourization of individual dyes was monitored for different time intervals until 48 h. Each experiment was carried out in triplicate.

2.6 Decolourization efficiency measurement

The samples were withdrawn from test tube after incubation and 1.5 ml was taken in fresh centrifuge tubes were rotated at 10000 rpm for 8 mins in a Micro centrifuge (MIKRO 120, Hittich ZENTRIFUGEN, Germany) at room temperature. The decolourization efficiency was determined by measuring the absorbance of culture supernatant at 595.5 nm using a T60 UV-Visible Spectrophotometer (PG-INSTRUMENTS, UK). This absorbance was compared with standard curve plotted using different concentrations of experimental dyes. From the standard curve the concentration of residual dye was measured. Then the efficiency of colour removal was expressed as the percentage ratio of the decolourized dye concentration to that of initial one based on the following equation: [25].

$$\text{Decolourization (\%)} = \frac{\text{Initial dye concentration} - \text{Residual dye concentration}}{\text{Initial dye concentration}} \times 100$$

III. Results And Discussion

3.1 Identification of the bacterial isolates

Isolation of dye decolourizing bacteria was carried out from textile effluent samples collected from the sites contaminated with wastewater from dyeing houses of Jaron Bazar. Five bacterial isolates with efficient decolourization ability against Cibacron Red FN-R dye were isolated using the technique described at section 2.2.

Selected isolates were identified on the basis of their morphological, cultural, physiological and biochemical characters. The test results are presented in Table-1. All the isolates recovered from textile effluent showed similar colony characteristics on Nutrient Agar and PEMBA (Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar). The morphological characteristics of all isolates were identical i.e. Gram positive rods (Fig. 1; left), arranged singly, in pair and sometimes in long chains (B5, B9) and motile. They contained central (A2, B6, C2) and sub-terminal (B5, B9) spores, isolate A2 and B6 were found to have para-sporal body (Fig. 1; right). Toxin-containing para-sporal bodies are present in *Bacillus thurengiensis* (Bt) when it observed under phase-contrast microscope [26].

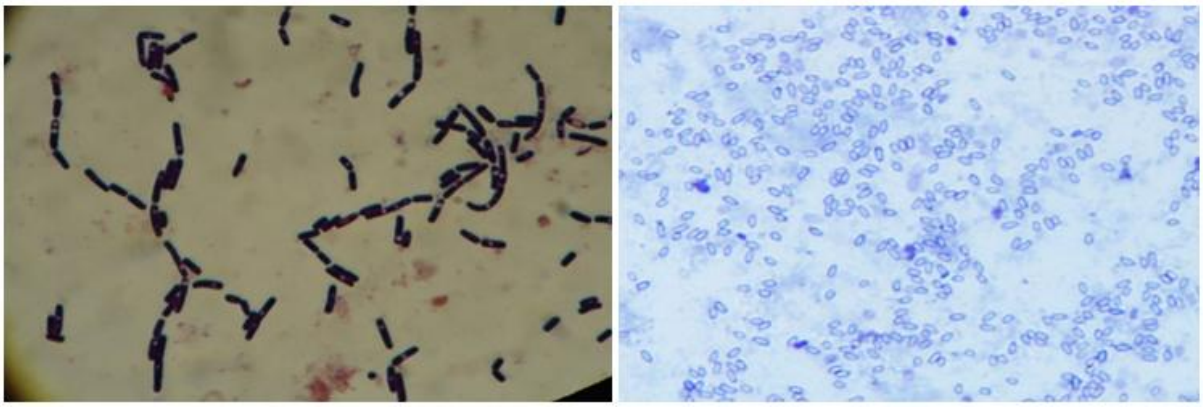

Figure 1: Gram staining (left) and Para-sporal body staining (right) of the bacterial isolate (B6) exhibiting vegetative cells and para-sporal bodies in 100 x magnification.

Physiological characters of the selected bacterial isolates were observed by subjecting those isolates at different temperatures and different Nacl concentrations. All isolates showed good growth at 20-40 °C but there was no growth above 45 °C and bellow 10 °C. With the increase of temperature from 20° C the isolates started to grow and the highest growth was observed at 30-35 °C for all isolates. The selected bacterial isolates were able to grow well at 2% NaCl solution and growths were decreased with the increase of salt concentration and finally growth disappeared at 6% salt concentration**.**

Following the comparison of the test results with descriptions in Bergey's Manual of Determinative bacteriology [24], and the isolates were found closely related to *Bacillus thuringiensis* (Isolate A2, B6), *Bacillus badius* (Isolate B5, B9), *Bacillus aneurinolyticus* (Isolate C2).

3.2 Effect of temperature on dye decolourization

The isolates were used to study the decolourization of Novacron Orange FN-R (Fig. 2A), Novacron Red FN-R (Fig. 2B), and Terasil Green (Fig. 2C), at different temperatures (20 °C, 30 °C, 35 °C and 40 °C). After 48 h of incubation, the decolourization activity was measured at certain time interval (after 18, 24, 42 and 48 h). All five isolates showed approximately similar decolourization pattern for these three experimental dyes. It was noticed that the decolourization percentages of dyes were increased with the increase in temperature from 20 to 35 °C and it was slightly decreased (which is not significant) with further increase in temperature up to 40 °C. The highest decolourization (91.33%) was observed at 35 °C for the Terasil Green dye by isolate A2. Therefore, the optimum temperature for the decolourization of these three experimental dyes bye the selected bacterial isolates were found to be 30 to 35 °C. This could be due to the optimum temperature for the growth of *Bacillus sp.* are 30-35 °C. Similar to our result, the maximum decolourization of Reactive Red dye by mixed cultures was observed at 35 °C reported by Cetin and Donmez [27]. *Bacillus sp.* was observed to perform good decolourization at 35 °C in static conditions [28]. The decolourization of Red RBN dye by *A. hydrophilla* in the range of 20–35 °C [29]. The bacterial consortium JW-2 showed maximum 93% decolourization of Reactive Violet 5R at 37 °C found by Moosvi *et al*. [30] and they also reported that, the decolourization rate of Reactive Violet 5R dye decreases further increase or decrease in temperature from optimum. The decolourization rate of azo dyes by many bacterial strains increases with increasing temperature up to the optimal temperature, within a defined range, and then there is a marginal reduction in the decolourization activity. The decrease in colour

removal activity at higher temperatures can be due to loss of cell viability or to the deactivation of azoreductase enzymes [31, 32].

Table-1: Morphological, Cultural, Physiological and Biochemical behaviour of selected isolates

Test parameters		**Biochemical Test Result**				
		Isolate A2	**Isolate B5**	**Isolate B6**	**Isolate B9**	**Isolate C2**
Colony on NA		Irregular, Cream, Raised, Smooth	Irregular, Cream, Raised, Smooth	Irregular, Cream, Raised, Smooth	Irregular, Cream, Raised, Smooth	Irregular, Cream, Flat, Rough
Colony on PEMBA		Irregular, Whitish, Rough, Bluish ppt	Irregular, Whitish, Rough, Bluish ppt	Irregular, Whitish, Rough, Bluish ppt	Irregular, Whitish, Rough, Bluish ppt	Irregular, Whitish, Rough, Bluish ppt
Gram Staining		+ve, Short rod, Single	+ve, Rod, Single, Long chain	+ve, Rod, Single Pair, Short chain (occasionally)	+ve, Short rod, Long chain, Single (occasionally)	+ve, Rod, Pair, Short chain
Spore Staining		Spore (Central)	Spore (Sub-terminal)	Spore (Central)	Spore (Sub-terminal)	Spore (Central)
Crystal morphology		Para-sporal body present	Para-sporal body absent	Para-sporal body present	Para-sporal body absent	Para-sporal body absent
Growth at different temperature (° C)	4	-	-	-	-	-
	10	-	-	-	-	-
	20	+	+	+	+	+
	25	++	++	++	++	++
	30	+++	++++	+++	++++	+++
	35	++++	++++	++++	++++	++++
	40	+++	+++	+++	+++	+++
	45	+	+	+	+	+
	50	-	-	-	-	-
Growth at different Nacl Concentrations (%)	2	+++	+++	+++	+++	+++
	4	+	++	+	++	+
	6	-	-	-	-	-
Indole		-ve	-ve	-ve	-ve	-ve
Methyl Red (MR)		-ve	-ve	-ve	-ve	-ve
Voges-Proskauer (VP)		-ve	-ve	-ve	-ve	-ve
Citrate utilization		-ve	-ve	-ve	-ve	-ve
Gelatin liquefaction		+ve	+ve	+ve	+ve	+ve
H_2S production		-ve	+ve	-ve	-ve	-ve
Nitrate reduction		+ve	+ve	+ve	+ve	+ve
Urease		-ve	-ve	-ve	-ve	-ve
Egg yolk test		+ve	+ve	+ve	+ve	+ve
Proteolysis		-ve	-ve	-ve	-ve	-ve
Arabinose fermentation		-ve	+ve	-ve	+ve	+ve
Rhamnose fermentation		-ve	-ve	-ve	-ve	-ve
Trehalose fermentation		-ve	-ve	+ve	-ve	-ve
Melibiose fermentation		-ve	-ve	-ve	-ve	-ve
Glucose fermentation		-ve	-ve	-ve	-ve	-ve
Xylose fermentation		-ve	-ve	-ve	-ve	-ve
Sucrose fermentation		-ve	-ve	-ve	-ve	-ve
Mannitol fermentation		-ve	-ve	+ve	-ve	-ve
Arginine		+ve	+ve	+ve	+ve	+ve
Oxidase		-ve	-ve	-ve	-ve	+ve
Catalase		+ve	+ve	+ve	+ve	+ve
Triple Sugar Iron Agar (TSIA)		ys/yb	rs/yb	ys/yb	ys/yb	ys/yb
Lysine Iron Agar (LIA)		ps/pb	ps/pb	ps/pb	ps/pb	ps/pb
Motility test		+ve	+ve	+ve	+ve	+ve
Starch hydrolysis		+ve	-ve	+ve	-ve	-ve
Isolates Identified*		***Bacillus thuringiensis***	***Bacillus badius***	***Bacillus thuringiensis***	***Bacillus badius***	***Bacillus aneurinolyticus***

Note: "+ve" indicates positive reaction, "-ve" indicates negative reaction, "-"indicates no growth, "+" indicates growth (+ = scanty, ++ = moderate, +++ = good, ++++ = heavy growth) "rs"= red slant, "y""= yellow butt, "ys"= yellow slant, "ps"= purple slant, "pb"=purple butt. *identification was done based on limited description in Bergey's Manual of Determinative Bacteriology [24].

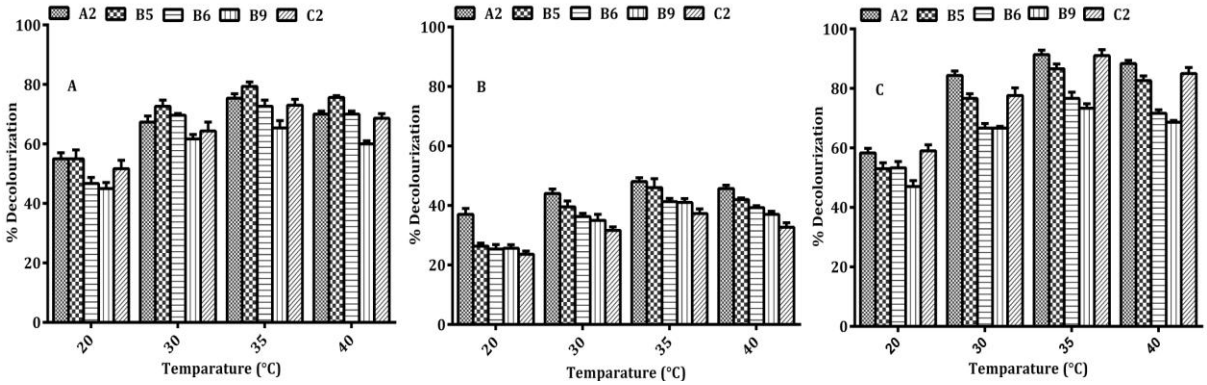

Figure 2: Decolourization of Novacron Orange FN-R (A), Novacron Red FN-R (B) and Terasil Green (C) by identified bacterial isolates at different temperatures (20 °C, 30 °C, 35 °C and 40 °C). The data is representative of three independent experiments after 48 h of incubation.

3.3 Effect of pH on Dye decolourization

Effect of pH on the decolourization of Novacron Orange FN-R, Novacron Red FN-R and Terasil Green was determined at pH 5, 6, 7, 8, 9 in the dye supplemented media M. Following the inoculation the experimental media M were incubated at 30 °C for 48 h. The isolates showed similar decolourization pattern at different pH (Fig. 3A, 3B, 3C). All isolates were able to decolourize the experimental dyes at a range of pH 5.0 to 9.0 but the maximum removal (98%) of Terasil Green by *Bacillus badius* (B5) was found at pH 7.0 after 48 h of incubation period. But there was no significant decolourization difference at pH 8.0 in contrast to pH 7.0. Further decrease in pH below 7.0 and increase in pH above 8.0 resulted in decreased percentage removal of dye. For *Bacillus thuringiensis*, *Bacillus badius* and *Bacillus aneurinolyticus* the optimum pH for decolourization were close to be 7.0 to 8.00 (shown in Fig. 3A, 3B, 3C after 48 h). For most of the dyes the optimal pH for decolourization was between 6.0 and 10.0 [33], which are similar to our findings. Decolourization of two reactive azo dyes Cibacron Black PSG and Cibacron Red P4B by *Bacillus cereus* was reported under aerobic conditions at pH 7.0 [34]. The results of our present study are in also good agreement with Tripathi A. and Srivastava S. K., [35] who achieved highest decolourization (90%) of Acid Orange 10 (250 mg L^{-1}) by *Pseudomonas putida* within 24 h at pH 7.0. Similarly, *Bacillus megaterium* gave highest decolourization of Turquoise Blue dye at pH 7.0 [36] and the optimal condition for the decolourization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain were at pH 7.0 and 35 °C [37]. The maximum decolourization of Methyl Red by *Micrococcus* strain R3 happened in pH range of 6.0–8.0 [38]. Decolourization of Remazol Black B by *Bacillus sp.* ETL-2012 was found in the pH range of 5.0–8.0 [39]. The optimum pH for decolourization of dyes is often at a neutral pH value or slightly acidic/alkaline pH and the rate of dye decolourization tends to decrease rapidly at strongly acid or strongly alkaline pH values [6]. The pH tolerance of decolourizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperatures [40]. The fact that *Bacillus sp*. isolated during the current study could decolourize reactive dyes in a relatively wide range of pH, make it suitable for practical bio-treatment of dyeing mill effluents.

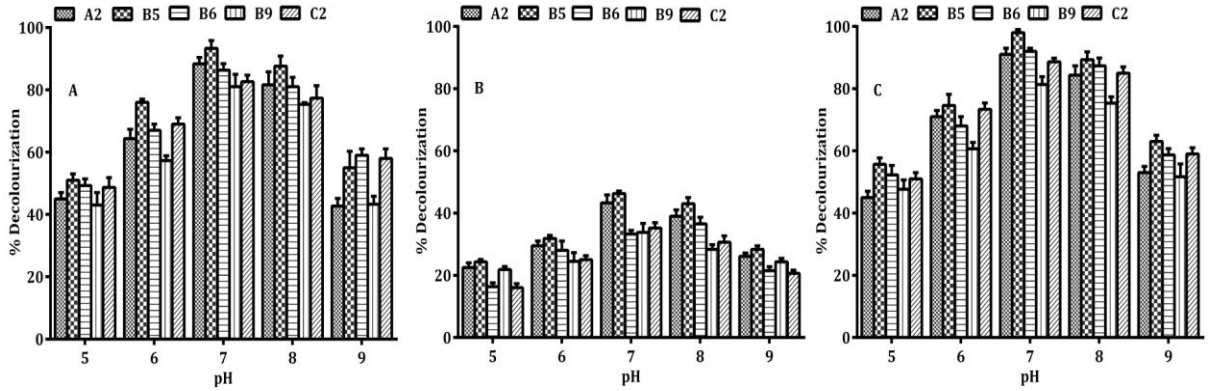

Figure 3: Decolourization of Novacron Orange FN-R (A), Novacron Red FN-R (B) and Terasil Green (C) by identified bacterial isolates at different pH (5, 6, 7, 8 and 9). The data is representative of three independent experiments after 48 h of incubation.

3.4 Effect inoculum concentration on dye decolourization

The effects of inoculum concentration (5-20% v/v) on decolourization of experimental dyes by the isolates at different time points were measured. It was observed that rate of decolourization was increased gradually with increased inoculum concentration. The decolourization percentage rapidly increased till 42 h, then became constant at all concentration of inoculum. The decolourization of Terasil Green by *Bacillus badius* after 48 h showed the best decolourization percentage (98%) was at inoculum size (20% v/v). The decolourization of the experimental dyes used in this study by selected isolates (A2, B5, B6, B9, C2) after 48 h did not show significant differences (Fig. 4A, 4B, 4C) at inoculum sizes 10%, 15%, and 20% (v/v) respectively, but decolourization percentage was less at inoculum size 5% (v/v). Hence, 10% (v/v) inoculum concentration selected as an optimum. Kumar K. *et al.* [41] reported similar finding when they used 10% (v/v) inoculum size during their work on aerobic decolourization of azo employing mixed culture. On the other hand, it was reported that a number of bacteria capable of aerobic decolourization of azo dyes which included *Bacillus subtilis, Bacillus megaterium, Bacillus thuringiensis, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus hominis* RMLRT03, *Staphylococcus aureus, Escherichia coli* [20, 22, 23,36, 37, 42].

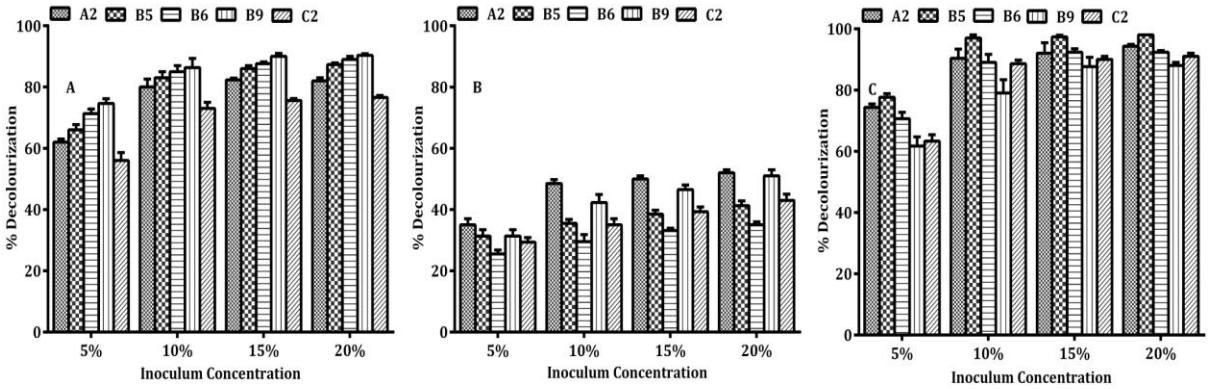

Figure 4: Decolourization of Novacron Orange FN-R (A), Novacron Red FN-R (B) and Terasil Green (C) by identified bacterial isolates at different inoculum concentration (5%, 10%, 15% and 20%). The data is representative of three independent experiments after 48 h of incubation.

3.5 Decolourization pattern of various textile dyes

Textile effluent consists of a mixture of various dyes. In our present study, the ability of *Bacillus sp.* (*Bacillus thuringiensis, Bacillus badius, Bacillus aneurinolyticus*) to decolourize different dyes were studied. *Bacillus sp.* was efficiently decolourize the three structurally different azo dyes used in this research within 48 h. The highest decolourization efficiency of 98% was recorded by isolate B5 (*Bacillus badius*) in Terasil Green. Besides, the highest decolourization efficiency recorded by the other experimental isolates in Novacron Orange FN-R and Novacron Red FN-R were 93.33% and 52% respectively. Our study suggested that the Novacron Red FN-R is more difficult to decolourize by selected bacterial isolates than other two experimental dyes. Similarly, Kalyani D. C. *et al.* [43] reported that the variation in the decolourization of different dyes might be attributable to the structural diversity of the dyes. It is also believed that anthraquinone dyes are more recalcitrant than azo dyes [44].

IV. Conclusion

The present study indicates that effluent adapted *Bacillus sp.* can be suitable for the decolourization of commonly used textile dyes in laboratory scale. The decolourization efficiency of isolated strains of *Bacillus sp.* against all the reactive dyes tested in this study was at satisfactory level which suggested that the isolates could be used to decolourize complex dyestuff effluent containing various reactive dyes. Moreover, further research on these strains is required to find efficient, cost-effective, and eco-friendly microbial solutions for the treatment of textile dyeing industrial effluents in large scale.

Acknowledgement

This study was supported by Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Qudrat-i-Khuda Road, Dhanmondi, Dhaka-1205, Bangladesh. The authors are grateful to Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), BCSIR, for providing the resources and requirements readily for the completion of this work.

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