



***Bacillus aryabhatai* SMNCH17-07 Strain: First Isolation and Characterization from Textile Wastewater with Evaluation of Its Decolorization Ability against Azo Dyes**

Safiye Elif Korcan¹  0000-0001-7875-5516

Kübra Çitekeçi²  0000-0003-3497-0606

Büşra Aydın²  0000-0002-1199-5600.

Ahmed Badri Abed²  0000-0002-7803-7917

Gülderen Uysal Akkuş³  0000-0002-0790-0170

¹ Uşak Health Training School, Uşak University, 1 Eylül Campus, 64300, Uşak, Türkiye

² Department of Molecular Biology and Genetics Department, Faculty of Arts and Sciences, Uşak University, 1 Eylül Campus, 64300, Uşak, Türkiye

³ Chemistry Department, Faculty of Science and Literatures, Afyon Kocatepe University, 03200, Afyon, Türkiye

Corresponding Author: Safiye Elif KORCAN, elif.korcan@usak.edu.tr

ABSTRACT

In this study, SA3 bacterial isolate, has been collected from textile wastewater area in industrial zone located in Uşak province, Turkey. Phenotypic identification and phylogenetic determination on the basis of partial 16S rDNA sequence comparisons indicated that this strain is 100 % *Bacillus aryabhatai* SMNCH17-07. The decolorizing ability of this strain was evaluated against CI Acid Blue 193 CI 15707 and CI Acid Red 88 CI 15620 dyes. The results showed that absorbance rate of Acid Blue 193 by this isolate was (33, 17%) after 216 hours. While Absorbance rate of Acid Red 88 by this isolate was (62,68 %) after 120 hours. According to FTIR spectrometer results of dyes adsorption it was found that bacterial retention sites were possibly aromatic and aliphatic (C = C, C = N, N = N) as well C-O groups. As the first report on the isolation of *Bacillus aryabhatai* SMNCH17-07 strain from textile wastewater with the evaluation of its ability to remove azo dyes, we suggest testing this bacterium as a low cost and ecofriendly bioremediator agent against further harmful dyes and pollutants

1. INTRODUCTION

Dyes are largely entered into various industrial products such as textile, medicinal compounds, body care preparations and etc [1-3]. Usually these dyes have a complex form due to their aromatic nature which increases resistance against biodegradation [4,5]. Around 10,000 diverse dyestuffs are applied in the fabric industry and about 7x10⁵ tons per year are manufactured worldwide [6].

Among these dyes, azo dyes are greatly entering into textile manufacturing, and form about 50% of the synthetic dyes used globally [7]. Azo dyes are poisonous, carcinogenic and genetic mutation inducer compounds and found to be harmful to some aquatic life and phototrophs in water due to reduced light penetration which can significantly affect

photosynthetic activity [3,8]. Adsorption, coagulation, flocculation, oxidation and electrochemical methods are usually used for treatment of dyestuffs from wastewater. But several disadvantages are related with these methods such as cost elevation, and the excessive sludge and by-products formation [3,9].

Conversely, biological processes can overcome physical and chemical methods disadvantages and can be more preferable for treating textile effluent due to their low cost, eco-friendly characters, and the low sludge production [10]. Bacterial strains isolated from dye-polluted sites have been demonstrated capability of decolorization and detoxification of azo dyes [11]. Decolorization of indigo carmine, Congo red, Reactive Black5 and reactive blue dyes have been evaluated by using *Streptomyces coelicolor*,

To cite this article: Korcan SE, Çitekeçi K, Aydın B, Abed AB, Uysal Akkuş G. 2022. *Bacillus aryabhatai* SMNCH17-07 strain: First isolation and characterization from textile wastewater with evaluation of its decolorization ability against azo dyes. *Tekstil ve Konfeksiyon* 32(2), 99-107.

Bacillus sp., *Shewanella oneidensis* WL7 and *Pseudomonas* sp. respectively [9,12-16].

Collecting air from the upper atmosphere by cryotubes was the step that led to the isolation of *Bacillus aryabhatai* for the first time [17]. Since that date few studies have been published on this bacterium and were concentrated in the field of bioremediation, health and the production of biopolymers. [18-22]. Recent published studies pointed that this bacterium can be used as a promise biocontrol source against *Bacillus glumae* [23] as well for producing and identification of biosurfactants associated with the biopharmaceutical products [24]. The attention on *B. aryabhatai* has been increased recently, due to the need of more studies on their genome, characteristics and abilities. Even the classification of this bacterium has been undergone to evaluation. Recent study suggested reclassifying of *B. aryabhatai* Shivaji et al. as a later taxonomic synonym of *Bacillus megaterium* [25].

This study has been notified and published in the list of changes in taxonomic opinion no.32 [26]. Recently Gupta RS et al. depended on the potent phylogenetic and molecular findings, suggested that 17 *Bacillus* species clades should be identified as new genera. This study reclassified *B. aryabhatai* to *Priestia aryabhatai* [27]. Studies about the ability of this bacterium to remove dyes are still very limited. *B. aryabhatai* DC100 ability to decolorize Coomassie Brilliant Blue, Remazol Brilliant Blue R and Brilliant Green was evaluated [28]. *B. aryabhatai* SMNCH17-07 strain was isolated for the first time in 2019 in Peru during the evaluation of the endophytic microbiota of rice cultivation against growth inhibition of *B. glumae* THT strain [29]. This strain was put in NCBI in 2019 under the accession number MK449444 [30]. To our knowledge there's a lack of studies about this strain and we couldn't find a published article pointed to the isolation of this strain from textile wastewater. Hence we are trying in our study to give more information about the characteristics and the abilities of *B. aryabhatai* SMNCH17-07 strain and indicate for the first time to the isolation of this strain from textile wastewater, as well

testing the ability of this strain to remove CI Acid Blue 193 CI 15707 and CI Acid Red 88 CI 15620 azo dyes.

2. MATERIAL AND METHOD

2.1 Material

Acid Blue 193, Acid Red 88, and Acid Yellow 42 used in the study were obtained from the textile factories of Uşak province (Table 1).

2.2 Method

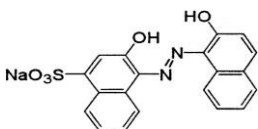
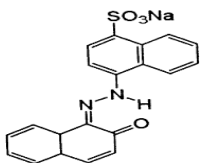
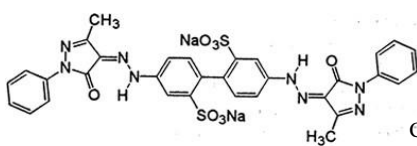
2.2.1. Determination of morphological, physical and biochemical properties of SA3 isolate

Samples of the untreated textile wastewater and activated sludge were collected from textile wastewater sludge area located in Industrial Zone (10 1 10 5) in Uşak province, Turkey. After samples being diluted at 65 ° for 45 minutes in a water bath, the strain has been isolated [31]. Gram staining preparations of the isolated strain have been performed. Their microscopic morphology and whether they formed spores were determined. Growth at different conditions, temperatures (18, 23, 37, 40 °C), pH (4, 7, 10) and 6.5% sodium chloride (NaCl) was evaluated. Also, starch hydrolysis, Voges Proskauer test (VP), catalase and citrate tests have been done.

2.2.2. Molecular identification

High Pure PCR Template Preparation Roche kit was used for DNA Isolation. Purity controls and quantification were performed spectrophotometrically using the Thermo Scientific-Nanodrop 2000c device. As a result, A260 / A280 ratio of 1.5µl DNA sample is aimed. Amplification of the 16S gene fragment was performed using the Taq DNA Polymerase Kit (HelixAmp™). PCR components and quantities of 1 µl primers sequence [11F (5'-GTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3')] has been used. The identification of the isolate was processed by analyzing the 16S rRNA gene sequence and comparing it with the existing 16S rRNA sequences present in Genbank and high scoring rRNA sequences in BLAST searches

Table 1. Acid Blue 193, Acid Red 88 and Acid Yellow 42 azo dyes used in this study.

Dye name	Open formula	Closed formula	Molecular weight
CI Acid Blue 193, CI15707		C ₂₀ H ₁₃ N ₂ NaO ₅ S	416.38
CI Acid Red 88, CI15620		C ₂₀ H ₁₃ N ₂ NaO ₄ S	400.38
CI Acid Yellow 42, CI22910		C ₃₂ H ₂₄ N ₈ Na ₂ O ₈ S ₂	758.69

2.2.3. Decolorization experiments

2.2.3.1. Detection of decolorization in solid media

After the cultivation of the obtained isolates with coloring medium (starch 10 g/L, nutrient broth 8 g/L, agar 20 g/L, dyestuff 0.15 g/L), decolorization has been evaluated based on the lightening of the color around the colonies after the incubation and the color of the colony that have been taken from the dye [32].

2.2.3.2. Decolorization experiment in broth medium

Bacterial isolates used in the study were inoculated into Luria bertani (LB) broth medium and incubated at 37 °C. 10 ml of 0.5 McFarland (1.5 10⁸ cells/mL) prebacterium culture was inoculated into 90 ml of LB broth medium containing dye. Incubation was carried out in a shaking at 37 °C and pH7 (optimal conditions for the isolate). Samples taken at regular intervals were centrifuged at 1600 rpm for 25 minutes. The maximum absorbance value of the supernants was read in the spectrophotometer.

The decolorization ability of isolated strain has been tested using 2 different dyes (CI Acid Blue 193, CI15707 and CI Acid Red 88). The peak value of CI Acid Blue 193 dye that we used was determined as 578 nm as a result of UV spectrophotometric measurement and the peak value of CI Acid Red 88 dye was determined as 504 nm. Measurements were made at different dye concentrations to create a standard curve. The decolorization percentage (%) results were calculated using the equations obtained from the standard curve [33]. Decolorization percentage was calculated according to the formula below.

Decolorization percentage (%) = [A₀ (initial absorbance) – A (Absorbance after decolorization)] / A₀ x 100 [34].

2.2.4. FTIR (Fourier Transform Infrared Spectrometer) color removal Analysis

2 mg of sample was weighed with a precision scale and 100 mg of KBr (Potassium Bromide) was added and the mixture was crushed in a mortar and a homogeneous mixture was obtained. This mixture was pressed into thin transparent discs and analyzed by FTIR (SHIMADZU IRAffinity-1S) [35]. Each new section and subsection should have a heading consisting of an arabic numeral followed by a period. Please a single space before and after the section title (see this template).

3. RESULTS AND DISCUSSION

3.1. Morphological, physiological and biochemical characters of isolate SA3

Isolate SA3 was observed as gram positive (Gr +) central spore forming bacillus. Isolated colony on nutrient agar medium showed a round, straight edges, cream color, shiny-oil and fluff shaped colony (Figure 1). Isolate SA3 used glucose as a carbon source, where it grows very well at temperatures between 18- 40 °C and pH values between

4-10. It has been observed that the isolate can also use fructose, arabinose and sucrose. In this study, it was determined that the optimum development of SA3 isolate was 37 degrees at pH7. Therefore, decolorization studies were carried out under optimum conditions. SA3 strain gave positive results for catalase, NaCl, Voges-Proskauer, starch hydrolysis assays and negative results for the citrate reaction (Table 2).

3.2. Molecular identification of SA3 isolate

3.2.1. Amplification of 16S rRNA Genes by Polymerase Chain Reaction (PCR)

The SA3 isolate yielded by using primers 11F and 1492R and were clearly determined to have bacteria 16S rRNA

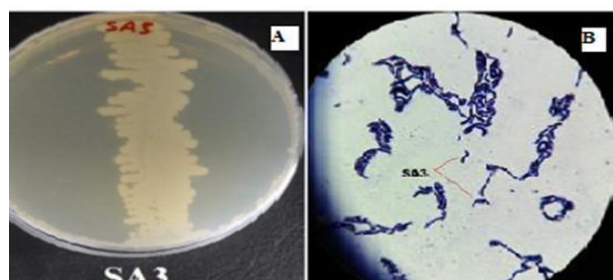


Figure 1. Colony appearance of isolated strain SA3 on nutrient agar (A), and microscopic appearance on 400x (B).

Table 2. Morphological, physiological and biochemical properties of SA3 isolate

Morphological		
Microscopic morphology	Gr (+)	
Spore	Bacil	
Colony color	Central Cream	
Physiological and biochemical properties		
pH	4	+
	7	+++
	10	++
Temperature (°C)	18	+
	23	++
	37	+++
	40	+
Sugar tests	Glucose	+++
	Fructose	++
	Arabinose	++
	Sucrose	++
Biochemical tests	Catalase	+
	Citrate	·
	VP	+
	NaCl	+
	Starch hydrolysis	*

(+) moderate, (++) good, (+++) very good growth

3.2.2. Sequence analysis of 16S rRNA genes

BLAST results, similarity ratio of the isolate obtained showed that SA3 isolate belong% 100 to *B. aryabhatai* (Table 3).

Table 3. Blast result of isolate SA3

Isolate no.	Length of sequence	Number of matched bases	Nearest in the gene bank	NCBI accession no.
SA3	2134	1155/1155	% 100 <i>Bacillus aryabhatai</i> strain SMNCH17-07	MK449444.1

3.3. Decolorization results

3.3.1. Decolorization results in solid media

Growth results of the SA3 isolate in acid yellow, Acid Red 88 and Acid Blue 193 dyes showed a positive result of adsorption for the three dyes (figure 2).

3.3.2. Determination result of the standard curve of Acid Blue 193 and Acid Red 88 Azo dyes

The peak value of CI Acid Blue 193 and CI Acid Red 88 dyes that determined as (578 nm) and (504 nm) as a result of UV spectrophotometric as well the measurements that made at different dye concentrations to create a standard curve are showed in (Figures 3 and 4)

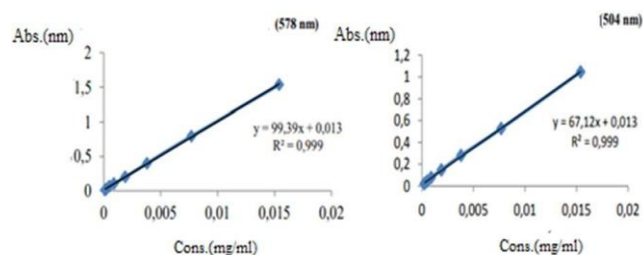
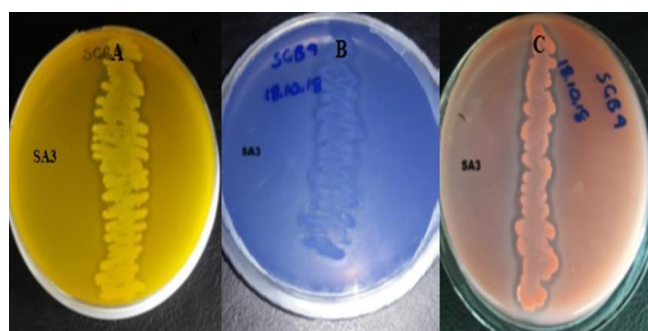


Figure 3. Acid Red 88 and Acid Blue 193 dye absorbance-concentration graph



(A) Adsorption on Acid Yellow media (B) Adsorption on Acid Blue 193 media and (C) Adsorption on Acid Red

Figure 2. Images of SA3 colonies formed on colored agar

3.3.3. Result of decolorization in LB Medium

The decolorization (%) rate of SA3 isolate used in this study against CI Acid Blue 193 and CI Acid Red 88 at (578 nm) and (504 nm) respectively and at 37 °C depending on time showed that the absorbance value of SA3 isolate against Acid Blue 193 continuously increased in the first 96th hour. It did not increase much later. While the result of the decolorization percentages of Acid Red 88 showed that the absorbance value increased up to (14%) in 24 hours, although the absorbance value increased slightly until the 216th hour (33%) (Table 4). The decolorization rate (%) of SA3 isolate against Acid Red 88 (504 nm) and Acid Blue 193 (578 nm) dyes depending on the time is given in (Figure 5).

Table 4. Spectrophotometer readings and decolorization rate (%) by SA3 isolate against Acid Blue 193 and Acid Red 88 dyes

Time (hour)	Acid Blue 193			Acid Red 88		
	Readings on spectrophotometer	Dye concentration	Absorbance (%)	Readings on spectrophotometer	Dye concentration	Absorbance (%)
Control	SA3	SA3	SA3	Control	SA3	SA3
0.80				0.50		
24	0.68	68.10	%14	0.37	25.05	%25
48	0.65	64.92	%17	0.26	17.26	%48
72	0.64	64.02	%18	0.23	15.25	%54
96	0.63	62.83	%20	0.20	13.37	%60
120	0.61	60.94	%22	0.18	12.43	%63
144	0.60	59.65	%24			
168	0.56	56.17	%28			
192	0.54	53.69	%32			
216	0.53	52.49	%33			

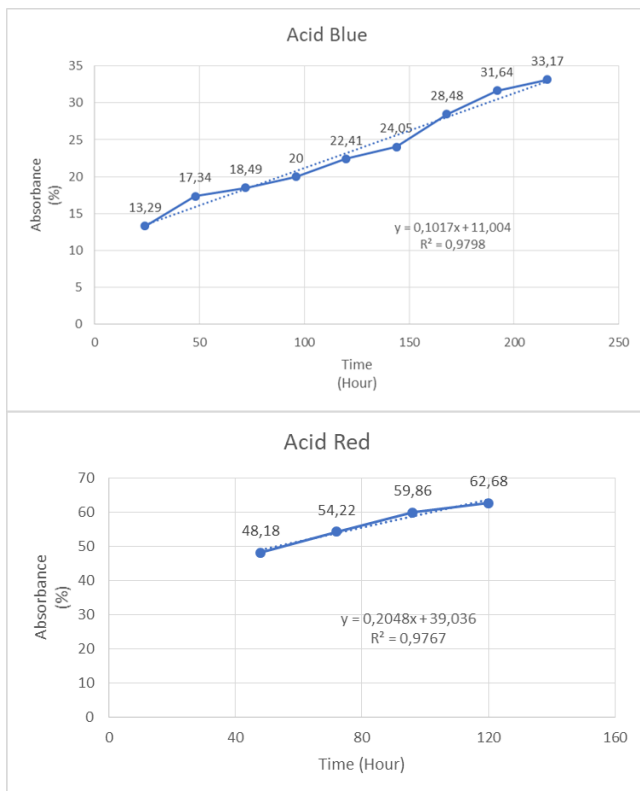


Figure 6. Percentage of decolorization of Acid Red 88 and Acid Blue 193 dye at optimum growth conditions

3.3.4. Results of color removal analysis with FTIR

FTIR analysis of isolate grown in Acid Red, Acid Blue 193 and Acid Yellow media are shown in (Table 5). Looking at the spectrum of the isolate grown in medium containing Acid Red 88 dye, secondary amine (N-H), (SO₃Na) and

carbonyl functional group (C=O) at approximately 3400 cm⁻¹, 1370 cm⁻¹, and 1700 cm⁻¹ were observed, respectively. Stretching peaks of (-N=N-) belonging to azo groups were seen at approximately 1500 cm⁻¹. In addition, stretching vibration peaks were seen for aromatic (C-H) bonds at 1640 cm⁻¹ and 700 cm⁻¹ and (C-N) bonds at 1200 cm⁻¹.

Growing in medium containing Acid Blue 193 dye showed that the spectrum belongs to the hydroxyl functional group (O-H), sulfonate functional group (S=O) and stretching vibration of azo groups (-N=N-) at approximately 3300 cm⁻¹, 1373 cm⁻¹, and 1520 cm⁻¹ respectively. Aromatic (C-H) bonds at 1640 and 700 cm⁻¹ and stretching vibration of (C-N) bonds at 1200 cm⁻¹ were observed.

In medium containing acid yellow dye, spectrum was belongs to secondary amine (N-H), carbonyl functional group (C=O), azo groups (-N=N-), sulfonate (SO₃Na) functional group and aromatic (C-H) bonds at approximately 3520 cm⁻¹, 1650 cm⁻¹, 1500 cm⁻¹, 1372 cm⁻¹ and 700 cm⁻¹ respectively. Stretching vibration peaks of (C-N) bonds were seen at 1200 cm⁻¹.

When looking at the spectra of the isolates interacted with the Acid Red 88 dye (peak number 2), it was seen that the peak of the secondary amine (N-H) disappeared at 3400 cm⁻¹, instead a wide peak of (O-H) stretching was formed. Note that the peak intensity of the carbonyl functional group (C=O) increased at 1700 cm⁻¹, and the peak of azo groups (-N=N-) at about 1500 cm⁻¹ and the peak of (C-N) bonds at 1220 cm⁻¹ disappeared. When compared to the isolate control spectrum given by the number 3 in the spectrum, it was determined that the isolate adsorbed the Acid Red 88 dye (Figure 6).

Table 5. FTIR analysis of SA3 isolate

Acid Red		Acid Blue		Acid Yellow	
Functional group	(cm ⁻¹)	Functional group	(cm ⁻¹)	Functional group	(cm ⁻¹)
Secondary amine (N-H)	3400	Hydroxyl group (OH)	3410	Secondary amine (N-H)	3400
(SO ₃ Na) group	1070	Sulfonate group (S=O)	1064	Carbonyl group (C=O)	1650
Carbonyl group (C=O)	1700	(-N=N-) group	1520	Azo group (-N=N-)	1520
Azo group (-N=N-)	1500	Aromatic bonds (C-H)	1649-700	(SO ₃ Na) group	1068
Aromatic bonds (C-H)	1640-700	(C-N) stretching	1396	Aromatic bonds (C-H)	1640-700
(C-N) stretching	1220			(C-N) stress vibration	1276

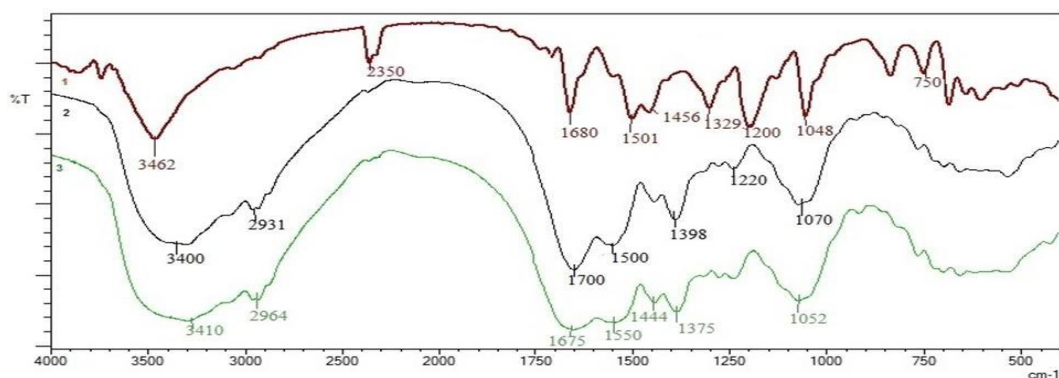


Figure 6. FTIR analysis of SA3 isolate Acid Red 88 (product) dye, (1) Acid Red 88, (2) Acid Red 88 dye adsorbed by the isolate (3) isolate itself

When looking at the spectra of the isolates interacted with Acid Blue 193 dye (peak number 2), the increase in the intensity of the peaks of 3410 cm^{-1} dehydroxyl functional groups (O–H), the formation of new peaks of the carbonyl functional group (C=O) at 1649 cm^{-1} were observed.

It was also observed that the peak of azo groups (--N=N--) at approximately 1520 cm^{-1} and the peak of (C–N) bonds seen at 1064 cm^{-1} disappeared. When compared to the isolate control spectrum given by number 3 in the spectrum, it was determined that the isolate adsorbed the Acid Blue 193 dye (Figure 7).

When looking at the spectra of the isolates interacted with the Acid Yellow dye (peak number 2), it was seen that the peak of the amine (N–H) at 3400 cm^{-1} disappeared and instead a wide peak of the hydroxyl functional group (O–H) was formed.

The intensity of the peak of the carbonyl functional group (C=O) increased at 1650 cm^{-1} , and the disappearance of the peaks of the azo functional groups (--N=N--) at about 1520 cm^{-1} indicated that the azo bonds were broken and interacted with the isolate from here. Again, looking at the spectrum, it was noteworthy that the stress vibration band of the C–N bonds seen at approximately 1068 cm^{-1} disappeared. When compared to the isolate control spectrum given by the number 3 in the spectrum, it was

determined that the isolates adsorbed the Acid Yellow dye (Figure 8).

4. DISCUSSION AND CONCLUSION

Wastewater of textile factories is released to the nature containing many toxic materials and dyestuffs including azo dyes which considered as harmful synthetic compounds [36]. Toxicity of azo dyes is attributed to their chemical composition which contains

--N=N-- bond besides the presence of aromatic rings which gives these dyes strong persistency in nature particularly in the water ecosystem [37,38].

Due to cost effectiveness and environmentally friendly approaches, microbial processes have been chosen to reduce the damage to humanity. *Bacillus* sp. is a remarkable bacterium with a high potential to produce various metabolites in different uses, it has been investigated whether they can potentially degrade crystal violet dye at different concentrations and time intervals. Fewer studies have reported that *Bacillus* species collected from textile industry wastewater can break down crystal violet dye used in these industries [39].

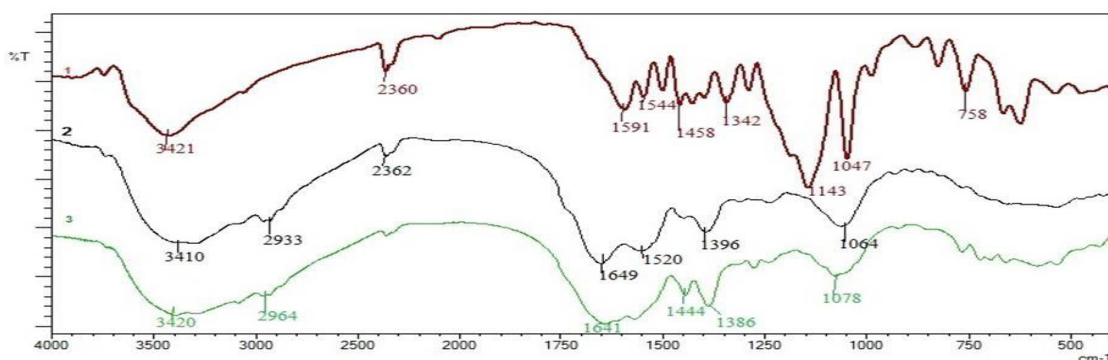


Figure 7. FTIR analysis of SA3 isolate and Acid Blue 193 (product) dye, (1) Acid Blue 193, (2) Acid Blue 193 adsorbing by the isolate), (3) isolate itself

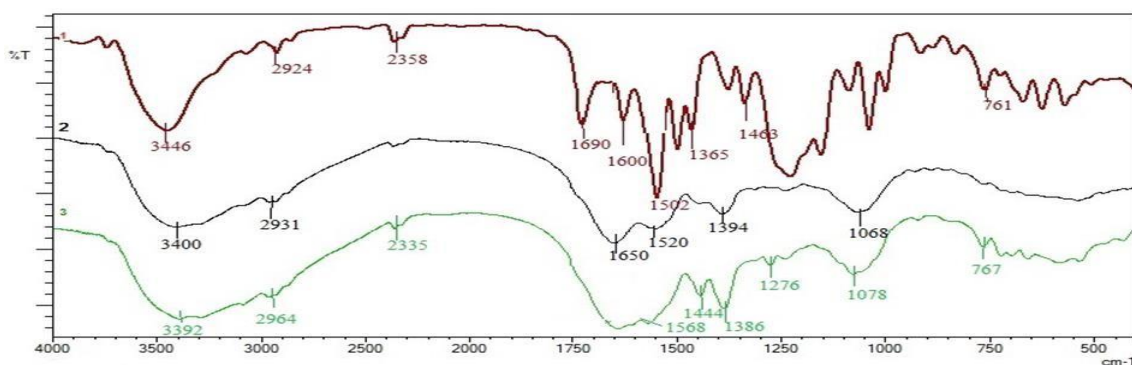


Figure 8. FTIR analysis of SA3 isolate and Acid Yellow (product) dye, (1) Acid Yellow, (2) Acid Yellow adsorbed by the isolate, (3) isolate itself

Recently, an interest towards *Bacillus aryabhatai* has been increased noticeably. Recent articles reported to the wide characters and abilities of novel strains belong to this bacterium. In our study SA3 strain which belongs to *B. aryabhatai* found to grow very well at (18-22) °C and the best growth value was in range between 37-40 °C. This result reveals the ability of this bacterium to live under a wide range of temperature. This can go with the findings that indicated to the ability of this bacterium to grow in different environments such as the stratosphere layer at an elevation between 27 and 41 km [17], the soil of Tibet at the elevation of 4123 m [40], the agricultural soil in India [41], and rhizosphere soil in South Korea [42,43].

Varieties in biochemical reactions have been observed also, since in our study SA3 strain revealed + result to Voges–Proskauer which is similar to *B. aryabhatai* EF 114313T, from Shivaji et al. (2009) which isolated from Upper atmosphere [17], but different from *B. aryabhatai* P1 isolated from poultry farm soil and from *Bacillus aryabhatai* FACU isolated from agroindustrial zone which found negative to Voges–Proskauer [44,45]. Evaluation of *B. aryabhatai* in degradation of pollutants including dye stuffs has been reported but in limited studies. This bacterium with its efficient enzymes was described as a multi-functions bacterium. It has been recommended in using for industrial purposes as a converter of lignocellulosic wastes into by products, a biodegrader of textile dyes and also as a biotransformer of effluents phenolics into add-value flavors and aromas [28,46,47]. Recent study proposed the usage of *B. aryabhatai* FACU3 as a bioremediator of glyphosate in pollutant sites [45].

Certain strains of *B. aryabhatai* has been tested against different types of dyes. These dyes included Direct Red-81 (DR-81) and Direct Orange-34 (DO-34) [44], Coomassie Brilliant Blue G-250 (CBB), Indigo Carmine (IC) and Remazol Brilliant Blue R (RBBR) [48], chrysoidine G, acid orange 74, carmine, crystal violet, etythrosine, reactive blue 19 and adizol black B [49]. Some studies reported that certain enzymes of *B. aryabhatai* found to be involvement efficiently in decolorizing of azo dyes [49,50].

The application of live cells is highly advantageous as both biosorption and biodegradation mechanisms may occur, often leading to enhanced dye removal. But the decolorization time will be long. Rapid decolorization of

dead cell lysates has lower saturation points due to the electrostatic interaction of cationic or anionic dye molecules with negatively charged functional groups present on biomass (e.g., amino, carboxyl, hydroxyl, and phosphate) [51]. For this reason, live bacterial isolate was used in our study.

In our study two new azo dyes have been tested which are CI Acid Blue 193 and CI Acid Red 88. Our results showed that the absorbance value of the CI Acid Blue 193 dye by SA3 isolate was 17.34% in the first 48 hours, but it was increasing in a low fixed degree until the 216th hour.

While the absorbance value of CI Acid Red 88 dye reached 48.18%, a very high increase, within 48 hours and the increase rate continued until the 120th hour.

This difference in absorbance values by SA3 isolate towards the two dyes depending on time is close to the findings of Rathod and Pathak (2017) who inferred that the absorbance rate (%) by *B. aryabhatai* P1 towards Direct Red-81 was 11.18% within 48 hours and the increase rate continued until the 120th hour which reached to 40.3%. While the absorbance rate (%) towards Direct Orange was 14.51% within 72 hours and was increasing in a low fixed degree until the 144th hour [44]. This indicates that the type of dye and time are factors that may determine the ability of *Bacillus aryabhatai* to removal dyes.

In conclusion we report in this study the first isolation of *B. aryabhatai* SMNCH17-07 strain from textile wastewater as well the evaluation of the ability of this strain to remove CI Acid Blue 193 and CI Acid Red 88 azo dyes. Showing the ability of this strain to remove azo dyes presents biological, low cost and ecofriendly strategy in treatment of harmful dyes. This can add more information about this bacterium which may permits for further studies to test wide range of dyes and pollutants. Moreover, studying the enzymes which involve in bioremediation processes and factors that enhance the activity of these enzymes are also demanded in future studies. Finally, novel strains belong to *Bacillus aryabhatai* are reporting continually and these strains which found in different habitats showed a wide characteristics and abilities under a different scales of conditions. All these facts about this species make it as a promising bacteria that can be applied alone or in synergism with other bacteria in bioremediation of many pollutants.

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