Pamukkale Univ Muh Bilim Derg, 22(6), 486-492, 2016

Pamukkale Üniversitesi Mühendislik Bilimleri Dergisi

Pamukkale University Journal of Engineering Sciences

Biosorption and decolourization of turquoise blue HFG by *Lysinibacillus sphaericus* LS-22 in aqueous solution

Turkuaz mavisi HFG'nin sulu çözeltide *Lysinibacillus sphaericus* LS-22 tarafından biyosorpsiyonu ve dekolorizasyonu

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Received/Geliş Tarihi: 06.04.2015, Accepted/Kabul Tarihi: 29.06.2015 doi: 10.5505/pajes.2015.00377
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Research Article/Araştırma Makalesi

Abstract

In this study, decolourization of Turquoise Blue HFG by Lysinibacillus sphaericus LS-22 was studied. And the effects of environmental conditions such as pH, dye concentrations, temperature, carbon and nitrogen sources on bacterial decolourization were determined. Dye concentration of 50 mg/L, pH 7.0 and 40 °C were found more effective conditions than the others tested. Compared to the carbon and nitrogen sources used, while sucrose and galactose stimulated decolourization activity, nitrogen sources inhibited. GC-MS and FT-IR results confirmed that one remarkable metabolite (Di-n-octyl phthalate) was detected at the end of degradation process. The results indicate that L. sphaericus LS-22 was effectively decolourized Turquoise Blue HFG.

Keywords: Turquoise blue HFG, Decolourization, Metabolite, *Lysinibacillus sphaericus*

Öz

Bu çalışmada, Turquoise Mavisi HFG'nin Lysinibacillus sphaericus LS-22 bakterisi tarafından dekolorizasyonu çalışılmıştır. pH, boya konsantrasyonu, sıcaklık, karbon ve azot kaynakları'nın bakteriyel dekolorizasyon üzerine etkisi belirlenmiştir. 50 mg/L boya konsantrasyonu, pH 7,0 ve 40 °C'nin test edilen diğer şartlardan daha etkili olduğu bulunmuştur. Kullanılan karbon ve azot kaynakları karşılaştırıldığında sükroz ve galaktoz dekolorizasyon aktivitesini teşvik ederken, azot kaynakları inhibe etmiştir. Degredasyon süreci sonunda, GC-MS ve FT-IR sonuçları dikkate değer bir metabolitin varlığını doğrulamıştır. Elde edilen sonuçlar L. sphaericus LS-22'nin Turquoise Mavisi HFG'yi etkili bir şekilde dekolorize ettiğini göstermiştir.

Anahtar kelimeler: Turquoise mavisi HFG, Dekolorizasyon, Metabolit, *Lysinibacillus sphaericus*

1 Introduction

Chemical dyes have important roles in water pollution. Synthetic dyes have been extensively used in the textile industry, agricultural research, paper production, cosmetics, leather dyeing, pharmaceutical and food technology [34],[35]. Because of their commercial value and extensive application, the uncontrolled discharge of synthetic dyes has led to contamination of groundwater resources and soil. Also, serious environment pollution has occurred [11],[20]. Synthetic dyes resistant to degradation due to their complicated molecular structures. Moreover, they are mutagenic and toxic [21],[37]. Traditional physical and chemical methods used for removal of dyes in wastewater are generally expensive; because of their limited applicability and the production of activated sludge, the newer treatment technologies need to be investigated. However, the biological remediation of textile effluents has recently offered an increasing attention, representing an attractive, cheap, environmentally friendly, and publicly acceptable alternative [2] to the physico-chemical methods [38]. Most of microorganisms such as cyanobacteria, bacteria, actinomycetes, algae, and fungi found in soil and water play an important role in the decolourization [10], [15], [19]. Undouble, the effectiveness of microbial decolourization depends on the adaptability and the activity of selected microorganisms. Fungi could be eliminated the synthetic dyes better than bacteria, but bacteria have the advantage due to their relatively short life cycle and faster decolourization process [41],[9]. Although many microorganisms belonging to genera such as *Staphylococcus* [9], *Bacillus* [33], *Aspergillus* [18], *Aeromonas* [6], *Proteus* [26] and *Pseudomonas* [13] can be decolorized of synthetic dyes under aerobic and/or anaerobic conditions in natural characteristic, environmental conditions on dye removal such as pH [39],[4] temperature [13],[39] presence or absence of oxygen [36] and presence of additional carbon and nitrogen sources [8] are great of importance.

Denizli is one of the textile centres of Turkey. As a result of widespread textile industries in Denizli, there is a large amount of effluents loaded with dyes. In this background, the present study was aimed to investigate the removal of one of the textile dye Turquise Blue HFG by *Lysinibacillus sphaericus* LS-22 strain isolated from Denizli, Turkey. The effects of parameters such as pH, temperature, initial dye concentration, and different carbon and nitrogen sources on the microbial decolourization rate by *Lysinibacillus sphaericus* LS-22 are studied quantitatively. Additionally, the decolourization products of dye were analyzed by FT-IR and GC-MS. Furthermore, the bio-sorption of dye by heat-inactivated *Lysinibacillus sphaericus* LS-22 cells was determined.

2 Materials and methods

2.1 Dye stock

The industrial quality Turquoise Blue HFG dye stock solution was obtained from Dystar Textile Co., Turkey. The powdered dyestuff was dissolved in distilled water at 1000 mg/L (w/v)

and sterilized by filter for the preparation of dye stock. Appropriate volumes of the stock dye were added to growth medium containing flasks.

2.2 Bacterial growth

Lysinibacillus sphaericus LS-22 used in the present work was obtained from the culture collection of the Pamukkale University, Bacteriology Laboratory. The strain was inoculated to a 250 ml Erlenmeyer flask containing 100 ml Tryptic Soy Broth (TSB; g/l: peptone from casein 17, peptone from soy meal 3, D(+) glucose 2.5, sodium chloride 5, di-Potassium hydrogen phosphate 2.5) medium and the culture was aerobically incubated with constant shaking at 125 rpm; culture growth was monitored by measuring optical density (OD) at 600 nm.

2.3 Decolourization experiments

The experiments were performed in 250 ml Erlenmeyer flasks containing decolourization medium (TSB). Turquoise Blue HFG was added to the medium and 10% (w/v) bacterium was inoculated into the medium. After incubation, the samples were withdrawn at different time intervals and analysed for decolourization efficiency. The aliquot was centrifuged at 14000 rpm to separate the bacterial cell mass. The decolourization rate was monitored spectrophotometrically by reading the decrease in absorbance (595 nm) of the dye in culture supernatant. Decolorizing activity is expressed in terms of percentage decolourization.

The effect of environmental factors such as initial dye concentration (25, 50 and 100 mg/L), pH (6.0, 7.0, 7.5, 8.0 and 9.0), temperature (40 and 50 ºC), carbon sources (galactose, sucrose, xylose, starch, glucose and lactose) and nitrogen sources (glycine, peptone from meat, aspartic acid, arginine, ammonium chloride, yeast extract, peptone from casein and meat extract) on bacterial decolourization were investigated for the specify the optimum conditions. Also, the growth of cells also routinely monitored by measuring optical density (OD) at 600 nm. The experiments were performed in duplicate and the mean values were taken into account.

2.4 Determination of decolourization efficiency

Decolourization extent was determined by measuring the absorbance of the culture supernatant at 595 nm using a UV-Vis Lange DR5000 spectrophotometer. The decolourization efficiency was calculated using the following equation:

$$\text{Decolourization efficiency (\%)} = 100x\ (OD_i - OD_t) / OD_i \qquad (1)$$

Where OD_i refers to the initial absorbance at 595 nm and OD_t refers to the absorbance measured in the degradation. The percentage of decolourization was measured at different time intervals. All decolourization experiments were performed in duplicate. Abiotic controls (without bacterium) were always included.

2.5 Biosorption

L. sphaericus LS-22 was incubated for 24 h in 1000 mL TSB medium and then centrifuged at 6000 rpm, +4ºC for 20 min. The obtained pellets were dried under aseptic conditions for 12-16 h at 80 ºC. After that, heat-inactivated cells were used as a bio-sorbent in the bio-sorption experiments. The bio-sorbents with the final concentration of 1 g cell/L were suspended in 100 mL of the dye solution (50 mg/L). The samples were incubated at 40 ºC and the dye concentration was periodically determined.

2.6 GC-MS and FT-IR analysis

Shimadzu GC-2010, gas chromatograph (Kyoto, Japan) equipped with MS-QP2010 plus mass spectrometer, AOC-20s auto sampler and AOC-20i auto injector were used for analysis. A 30 m x 0.25 mm Teknokroma 5MS fused silica capillary column (Teknokroma, Barcelona, Spain) was employed. The column temperature program was set as follows: 60 ºC hold for 1 min, at 10 ºC / min to 200 ºC hold for 15 min. The GC injector was held isothermally at 250 ºC with a splitless period of 3 min. All injection volumes were 1 µL in the splitless mode. The solvent delay time was set at 5 min. Helium was used as the carrier gas, at a flow rate of 10 mL min-1 by using electronic pressure control. The GC/MS interface temperature was maintained at 250 ºC. The MS was operated in electron impact (EI) ionization mode with electron energy of 70 eV and scan ranged from 50 to 500 amu (atom to mass unit) to determine appropriate masses for selected ion monitoring. Pelkin Elmer Spectrometer, BX FT-IR (Fourier Transform Infrared Spectroscopy) Pelkin Elmer, USA), was also used for the analysing biodegradation products.

3 Results

3.1 Effect of initial dye concentration

The effect of initial dye concentration on decolourization by using three different dye concentrations (25, 50, 100 mg/L) has been shown in Figure 1. In generally, *L. sphaericus* LS-22 bacterium could decolourize all concentrations of the dye with different rates. However, the maximum decolourization performance obtained with 50 mg/L dye concentration. At the first 24 h of incubation, colour reduction rates of *L. sphaericus* LS-22 in media containing 25, 50 and 100 mg/L dye were 26.94%, 62.39% and 41.66% respectively. Also, maximum decolourization rates were determined after 33, 24 and 45 hours of incubation for 25, 50 and 100 mg/L dye concentrations respectively.

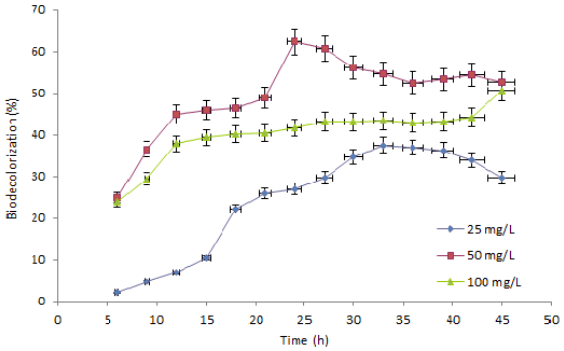

Figure 1: Effect of different initial dye concentrations on decolourization of Turquoise Blue HFG by *Lysinibacillus sphaericus* LS-22 at pH 7.0 and 40 ºC in TSB medium.

3.2 Effect of pH and temperature

Figure 2A shows the effect of the different pH values of the medium to the bacterial decolourization containing 50 mg/L initial dye concentration. To determine a suitable pH value for the most efficient decolourization by live cells of *L. sphaericus* LS-22, pH 6.0, 7.0, 7.5, 8.0 and 9.0 were initially tested. Bacterial decolourization of Turquoise Blue HFG was occurred

over a broad pH range 6.0-9.0 but the best decolourization was obtained in neutral pH by the bacterium. The lowest decolourization was generally occurred at pH 6.0 and 9.0.

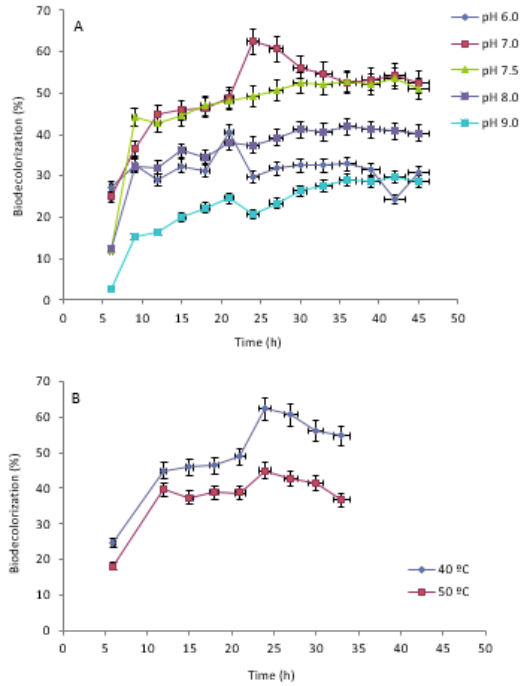

Figure 2: Effect of different pH, (A): and temperature, (B): Levels on decolourization of Turquoise Blue HFG with 50 mg/L dye concentration in TSB medium.

To determine the effect of different temperature on decolourization, *L. sphaericus* LS-22 bacterium was inoculated into the TSB medium containing 50 mg/L dye at pH 7.0 and incubated at 40 and 50 ºC. and the maximum decolourization was observed at 40 ºC with the 62.39% at 24th hours of incubation. Consequently, colour removal was better at 40 ºC than 50 ºC (Figure 2B).

3.3 Effect of different carbon and nitrogen sources

The effects of carbon sources such as galactose, sucrose, xylose, starch, glucose and lactose on decolourization were tested; results are given in Figure 3. When the carbon sources were added in the decolourization medium, the colour removal rates were increased. Especially, sucrose and lactose enhanced the decolourization rate of *L. sphaericus* LS-22 bacterium. But, interestingly starch was slightly decreased the decolourization of Turquoise Blue HFG compared to control (without carbon sources). At 50 mg/L dye concentration, maximum colour reduction was observed in the control at 62.39%. However, when sucrose and lactose was added in growth medium, colour reduction increased to 71.30% and 70.46% respectively.

Also, the effect of the nitrogen sources was studied. Eight nitrogen sources (glycine, peptone from meat, asparagine, arginine, ammonium chloride, yeast extract, peptone from casein and meat extract) were tested for decolourization of *L. sphaericus* LS-22 and none of the tested nitrogen sources were not increased the decolourization rate until 45th hours of incubation, compared to control (Figure 4).

Additionally, experiments with heat-inactivated cells of *L. sphaericus* LS-22 showed that bio-sorption onto cell material had a negligible impact for the loss of Turquoise Blue HFG from solution (Data not shown).

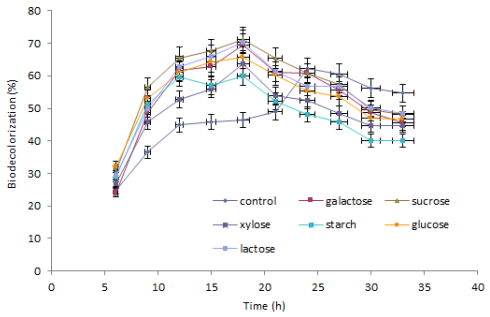

Figure 3: Effect of different carbon sources (1 g/L) on decolourization of Turquoise Blue HFG with 50 mg/L dye concentration in TSB medium (at 40 ºC and pH 7.0).

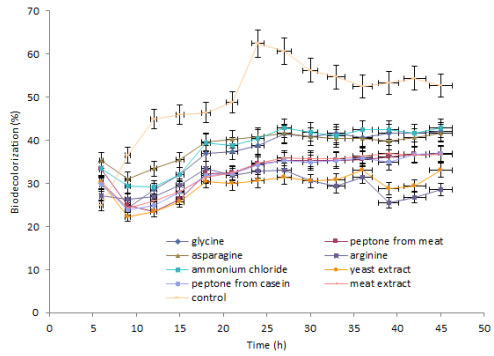

Figure 4: Effect of different nitrogen sources (1 g/L) on decolourization of Turquoise Blue HFG with 50 mg/L dye concentration in TSB medium (at 40 ºC and pH 7.0).

3.4 Analysis of metabolites

The FT-IR spectra of pure Turquoise Blue HFG and after decolourization were recorded in Figure 5. The spectrum of pure dye showed the characteristic absorption peaks at 1559.44 cm^{-1} (aromatic ring, C=C stretching), 2925.22 cm^{-1} (C-H stretching), 2362.16 cm^{-1} (C=N stretching) and 1193 cm^{-1} (C-N bending). Addition, the strong peak at around 3400 cm^{-1} was assigned to the stretching of N-H.

Compared with the FT-IR spectrums of pure dye (Figure 5A) and of after decolourization (Figure 5B) it is showed that the peak intensity is reduced at 2925 cm^{-1}. The peaks at 1193 cm^{-1} and 2362.16 cm^{-1} weren't observed of C-N stretching. Also, new hydroxyl groups occured after biodegradation. The strong peak (N-H stretching) at around 3400 cm^{-1} wasn't observed and OH peaks were appeared instead of it (Figure 5B).

Otherwise; the strong peak 2361.03 cm^{-1} was observed of CO_2 band. The peak at 1643.79 cm^{-1} was observed of C=O stretching.

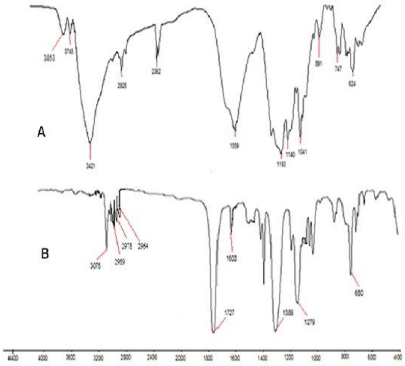

Figure 5: FT-IR Spectrums, A: pure Turquoise Blue HFG, B: after decolourization by *Lysinibacillus sphaericus* LS-22 at optimum conditions.

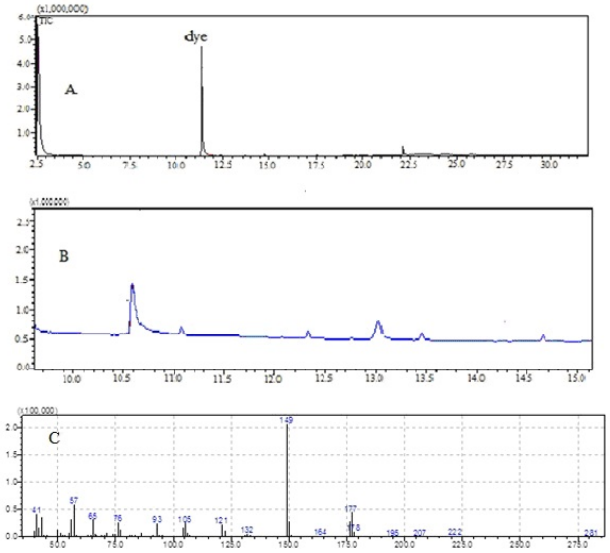

Figure 6: GC chromatograms, A): GC chromatogram of pure Turquoise Blue HFG, B): GC chromatogram of decolourization, C): Mass spectrum of Di-n-octyl phthalate (metabolite).

The purpose of GC-MS analyses is to identify some degradation or biotransformation products produced from the degradation of the Turquise Blue HFG. Turquise Blue HFG in the samples was identified by retention time. In the GC analysis, retention time of Turquise Blue HFG was detected as 11.667 min. According to the results, Di-n-octyl phthalate metabolites observed approximately at 10.5 min by GC after biodegradation process (Figure 6).

4 Discussion

It is known that bacterial decolourization is related to multiple factors such as salt, temperature, pH, and dye concentration, presence of carbon and nitrogen sources. Because of this, it is important that determining optimal environmental conditions to obtain efficient decolourization potential. In this paper, we reported Turquoise Blue HFG decolourization by thermal bacterium *Lysinibacillus sphaericus* LS-22 and determined the effect of environmental factors on decolourization.

As known, effect of the initial dye concentration is an important step in decolourization. So, three different dye concentrations (25, 50, 100 mg/L) were used to determine the best decolourization ability of *L. sphaericus* LS-22 on Turquoise Blue HFG (Figure 1). In dye concentration of 25 mg/L, dye removal efficiency varied from 36.91%-2.07%, where as in 100 mg/L dye concentration it range from 50.62%-23.95%. The highest decolourization of HFG by *L. sphaericus* LS-22 was obtained in the presence of 50 mg/L dye concentration (62.39%-24.86%). With increase in initial dye concentration, the percentage removal of Turquoise Blue HFG decreased. A similar trend was also reported in bacterial consortium [27] and *Citrobacter* sp. CK3 [39]. When a high concentration dyestuff is used to colour removal, dye decolourization can be strongly inhibited because of the toxic effect of dye on the degrading microorganisms [28]. Therefore, the high dye concentration is caused the decrease in decolourization efficiency at high dye concentrations [29].

The initial pH of the medium has a major effect on the efficiency of dye decolourization and also plays a crucial role in decolourization. So, many researchers have investigated the optimum pH values for bacterial dye removal. Hussain et al. [13] proved that decolourization of Reactive Black-5 is decreased at the extreme pH values (5.0, 6.0 and 9.0). In *Citrobacter* sp. CK3, the optimum pH values were 6.0 and 7.0 for decolourization of Reactive Red 180 and much lower decolourization was showed in strongly acidic (at pH 4) and strongly alkaline (at pH 12) conditions [39]. The variation observed in optimal pH indicates that it is important to individually determine the optimum pH value in different cultures and to modify the pH in order to achieve maximum bacterial decolourization. Therefore, we determined the effect of pH variation on decolourization at pH levels of 6.0, 7.0, 7.5, 8.0 and 9.0 in the present study. It was observed that the decolourization ability of *L. sphaericus* LS-22 was affected from pH change (Figure 2A). Generally, the decolourization was very little effected by altering the pH within a range of 7.0-7.5. *L. sphaericus* LS-22 decolourized (62.39% and 53.55%) the Turquoise Blue HFG after 24 and 42 h of incubation at pH values of 7.0 and 7.5, respectively. However, at pH 6.0, 8.0 and 9.0 the percentage removal of the dye of this strain was 40.46%, 41.85% and 29.81% after 21, 36 and 42 h, respectively. According to these results, the best colour removal activity was obtained at pH 7.0. Especially in acidity (pH 6.0) and alkalinity (pH 9.0), the percentage decolourization of HFG decreased significantly. It is also confirmed by Saratale et al. [30] and Wang et al. [39] that the dye reduction tends to decrease at strongly acid or alkaline pH.

Figure 2B shows the effect of temperature on decolourization of Turquoise Blue HFG by *L. sphaericus* LS-22. The decolourization percentage was decreased with increase in temperature (from 40 ºC to 50 ºC). In other words, the colour removal rate at 40 ºC was higher than 50 ºC. The rate of decolourization ranged from 24.86% to 62.39% at 40 ºC and from 18.18% to 45.01% at 50 ºC. The optimum temperature showed was 40 ºC for this bacterium. The highest dye removal was observed after 24 h of incubation (62.39%) at 40 ºC. So, decolourization efficiency at 40 ºC was better than at 50 ºC. This was also confirmed by Pearce et al. [28]. According to Pearce et al. [28], decrease in decolourization percentage at higher temperature can be connected with the loss of cell viability. Bacterial decolourization is an enzymatic reaction and as is shown temperature affect enzyme activity and cell survival [3]. Effects of temperature on dye reduction are investigated by some researchers. For example Wang et al.

[39] reported that *Citrobacter* sp. CK3 showed strong decolorizing activity from 27 ºC to 37 ºC and at 42 ºC decolourization activity was decreased. Another bacteria *Pseudomonas* sp. RA20 efficiently decolourized the Reactive Black-5 at 25 ºC [13]. We can say that dye removal ratio by *Lysinibacillus sphaericus* LS-22 was moderate when compared with the other studies (Table 1).

During the past few years, several bacterial strains have been isolated that can aerobically decolourize synthetic dyes. Bacteria cannot utilize dye as the growth substrate. Because of this, many of these strains require the addition of carbon and nitrogen sources in decolourization medium [34]. Therefore the bacterial decolourization showed differences when the bacteria were grown in the presence of various types of carbon or nitrogen sources. In generally, carbon sources showed significant influence on decolourization in bacterium. The maximum decolourization yield was found in presence of sucrose and lactose after 18 h of incubation (71.30% and 70.46% respectively) (Figure 3). Galactose, xylose, starch and glucose were slightly decreased the colour reduction rate after 18 h of incubation time compared to sucrose and lactose.

Table 1: Comparison of the decolorization performance of different bacterial strains with *Lysinibacillus sphaericus* LS-22.

Strain	Dye and concentration	Condition [pH, temp. (°C), agitation]	Time (h)	References
Bacillus sp. YZU1	Reactive Black 5 (100 mg/l)	7.0, 40, static	120	[40]
Lysinibacillus sp. AK2	Metanil Yellow (200 mg/l)	7.2, 37, static	12	[1]
Lysinibacillus sp. KMK-A	Orange M2R (1000 mg/l)	7.0, 37, static	72	[5]
Bacillus fusiformis KMK5	Acid Orange 10 (1500 mg/l)	9.0, 37, static	48	[16]
Micrococcus glutamicus NCIM-2168	Reactive Green 19 A; (50 mg/l)	6.8, 37, static	42	[32]

Similarly, the performance of *Lysinibacillus* sp. RGS in decolorizing Remazol Red in the presence of glucose and starch was not remarkable effective [31]. A commercial tannery and textile dye, Navitan Fast blue S5R was decolourized by *P. aeruginosa* in the presence of glucose under aerobic conditions [31]. However in another study, glucose, sucrose, and glycerol were found as effective electron donors while acetate, citrate, and lactate were unfavourable electron donors for AQDS (anthraquinone-2, 6-disulphonate, humus) reduction by *Planococcus* sp. MC01 humus-reducing facultative anaerobe strain. Also this bacterium showed high decolorizing activity of Orange I at the optimal glucose concentration [7]. On the other hand there are many studies in the literature also about the effect of nitrogen sources on colour removal. For example, Modi et al. [22] indicated that peptone was the best nitrogen source for efficient reduction of Reactive Red 195 by the isolate *B. cereus* M1 among all nitrogen sources tested. Gurulakshmi et al. [12] reported starch and peptone to be the most effective carbon-nitrogen sources in decolourization. Junnarker et al. [14] reported that starch and casein to be the most effective carbon-nitrogen source in decolourization of Direct Red 81 by bacterial consortium. In the present study, addition of nitrogen sources seemed to be not effective to promote the decolourization. Percent decolourization of Turquoise Blue HFG by this strain decreased in the growth medium supplied with nitrogen sources (Figure 4). Ammonium chloride resulted in 42.78% maximum colour reduction while asparagine resulted in 41.55% reduction at 27th hours. These were followed by peptone from meat (36.64%), meat extract (36.93%) and glycine (41.66%) (Figure 4).

Due to detect the metabolites, last sample of depletion was analysed via GC-MS. In accordance with data, only one metabolite (Di-n-octyl phthalate) was detected (Figure 6). Thus, biotransformation of Turquoise Blue HFG was designated by this organism. Recently, the biotransformation of imidacloprid by *Pseudomonas sp.* 1G was reported, having 70% degradation efficiency within 14 days, via the formation of desnitro and urea metabolites [25]. Lade et al. [17] was reported that Vanishing of major peaks and formation of new peaks in the IR spectrum of consortium-AP metabolites suggests the biotransformation of dye into distinct metabolites. Phugare et al. [27] demonstrated that The GC-MS analysis showed the probable metabolites produced during the Red HE3B biotransformation process.

5 Conclusion

The effects of environmental factors (pH, temperature, and dye concentration, carbon and nitrogen sources) on decolourization to achieve maximum colour removal by *L. sphaericus* LS-22 strain have been determined in the present study. It has been found that xylose is the most effective carbon sources, which can be used for decolourization of Turquoise Blue HFG. Also sucrose, lactose and ammonium chloride combination has stimulated the decolourization although they decrease the decolourization efficiency when they used separately. The optimum pH was 7.0 and temperature was 40 ^{o}C and there is a negative correlation between initial dye concentration and bacterial decolourization. Additionally, the Turquoise Blue HFG was not biosorped onto cell surface by LS-22 bacterium. GC-MS and FT-IR results indicate that one remarkable metabolite (Di-n-octyl phthalate) was detected at the end of decolourization process. Consequently, Turquoise Blue HFG textile dye was bio-transformed by *L. sphaericus* LS-22.

6 Acknowledgments

The authors thank to Dr. Hatice ARDAG AKDOGAN (Pamukkale University) and Merve CANPOLAT (Pamukkale University) for providing GC-MS and FT-IR experiments.

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