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Biodecolourization and biotransformation of textile dyes remazol violet-5R and remazol brillant orange-3R by *Bacillus* sp. DT9 isolated from textile effluents

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Abstract

The present study aims to evaluate RV-5R and RBO-3R decolourizing potential of *Bacillus* sp. DT9 isolated from textile effluent in Denizli (Turkey). In present study, maximum dye-decolourizing efficiency of the culture was achieved at 25 mg l^{-1} concentration of RV-5R and 500 mg l^{-1} concentration of RBO-3R. While the optimum dye-decolorizing activity of DT9 was observed at pH 7.5 and 37 °C in sucrose for RV-5R (91.66% decolourization rate), 500 mg l^{-1} RBO-3R concentration by *Bacillus* sp. DT9 was decolourized at pH 9.0 and 30 °C in sucrose/peptone containing medium (98.41% decolourization rate). In other step of study, DT9 was immobilized in Ca-alginate. According to immobilization results, the percentage of decolourization of RV-5R was found very similar to cell free result. But, the percentage of decolourization of RBO-3R decreased 30.0%, when DT-9 cells were immobilized in Ca-alginate. Metabolites of the RV-5R and RBO-3R biodegradation were analysed via ESI-TOF/MS analysis at the end of decolourization process, and the biotransformation and dimerization was confirmed.

Keywords: *Bacillus,* Remazol Violet-5R, Remazol Brillant Orange-3R, Decolourization, Dye removal

1. Introduction

Synthetic dyes have extensive application in textile industry because of their commercial importance and contaminated groundwater resources and soil. These dyes are discharged directly into the environment together with wastewater (Robinson *et al*., 2001; Deng *et al*., 2008). Additionally, trace amount of dyes (10-15 mg l^{-1}) are highly visible, affecting water recreational value, light penetration in water and as a consequence reduced photosynthesis and dissolved oxygen (Dafale *et al*., 2010). It is known that traditional wastewater treatment technologies are ineffective for handling wastewater of synthetic textile dyes because these pollutants are stable chemically. Hence, the removal of synthetic dyes is to be solved an important problem. Contrary to traditional wastewater treatment technologies, biological methods are eco-friendly. Most studies are focused on biologic decolourization and bacterial decolourization attracts great interest since it is a highly efficient technique. Several researchers have also reported to the organisms, which are capable of decolourization dyes under various environmental conditions (Guo *et al*., 2007; Wang *et al*., 2009; Olukanni *et al*., 2010; Arar *et al*., 2014; Mukherjee and Das 2014, Vijayaraghavan and Shanthakumar, 2015).

In this paper, decolourization of Remazol Violet-5R (RV-5R) and Remazol Brillant Orange-3R (RBO-3R) was studied by *Bacillus* sp. DT9 isolated from textile effluents. The different parameters such as initial dye concentration, pH, temperature, and carbon and nitrogen sources were tested quantitatively. In addition, decolourization efficiency of cell free of DT9 was compared to immobilized cells in Ca-alginate matrix. Also, laccase activity was determined and the metabolites in bio-decolourization process were detected by ESI-TOF/MS analysis.

2. Materials and methods

2.1. Dye stock, isolation, and bacterial growth

Remazol Violet-5R (RV-5R) and Remazol Brillant Orange-3R (RBO-3R) were kindly supplied from Dystar Textile Co., Turkey. Stock dye solution was prepared at concentration of 1000 mg l^{-1} (w/v) sterilized by filter and added to the media in all manipulations. *Bacillus* sp. DT9 was isolated from textile effluent in Denizli (Turkey). The samples were inoculated on solid agar containing 100 ppm dye. After 2 days of incubation, the bacterial colonies of forming a clear zone on the medium were selected and stocked. Gram and endospore staining were performed to confirm to rod-shaped bacteria. Tryptic Soy Broth (TSB) (Sigma, 22092) and Tryptic Soy Agar (TSA) (Sigma, 22091) were used in experiments.

2.2. Decolourization experiments and conditions

10% (w/v) bacterium was inoculated in TSB medium contained dye and flasks were incubated at 37 °C on a

rotatory shaker (125 rpm). The culture was centrifuged at 14000 rpm to separate the bacterial cell mass. The decolourization was quantified by measuring the decrease in absorbance of the dye using UV-Vis Spectrophotometer (HACH Lange DR5000). Dyes of RV-5R and RBO-3R had λmax values of 560 and 494 nm, respectively and then dye decolourization (%) was calculated. The initial dye concentration, pH, temperature, carbon and nitrogen sources were investigated to determine maximum dye removal conditions. The experiments were performed in duplicate and the mean values were taken into account.

2.3. Immobilization

Bacillus sp. DT9 was cultivated in best decolourization conditions (25 mg l^{-1} RV-5R, pH 7.5, sucrose, 37 ◦C and 500 mg l^{-1} RBO-3R, pH 9, sucrose, 30 ◦C). After cultivation, the cells were centrifuged at 6000 rpm for 20 min and washed twice with sterile water. 6 ml of 2% (w/v) sodium alginate solution prepared in distilled water and mixed with the pellets (0.5 and 1.0 g). In order to obtain the beads, the slurry was extruded through a syringe into $CaCl_2$ solution (2% w/v) and kept at 4ºC for 4 h. After that, beads were washed with sterile physiological water (Puvaneswari *et al*., 2002) and used for inoculation of 50 ml of the decolourization medium.

2.4. Screening of laccase by plate assay

Plate assay method was performed to confirm presence of laccase (More *et al*., 2011). 2,2´-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS, A1888-Sigma) was used as substrate. The presence of an intense bluish-green colour was considered as a positive test for laccase activity.

2.5. Preparation of cell-free extract and laccase activity

Bacillus sp. DT9 was incubated in optimal decolourization conditions. Pellet was collected by centrifuged at 8000g for 20 min and suspended in 50 mM potassium phosphate buffer (pH 7.4). After sonication, this cell-free extract was used to determinate laccase activity (Dawkar *et al*., 2010). The activity was determined by measurement of the enzymatic oxidation of ABTS at 420 nm (ε = 3.6 × 10^4 cm^{-1} M^{-1}). The reaction mixture contained 0.3 mL of 1.0 mM ABTS, 2.4 mL of 0.1 M sodium acetate buffer (pH 4.5), and 0.3 mL cell-free extract and incubated for 5 min. The activity was expressed as nmol mL^{-1}.

2.6. ESI-TOF/MS analysis

All ESI-TOF/MS analysis was performed by TUBITAK (Gebze, Turkey). Microtof II mass spectrometer (Bruker Daltonics) was used for the determination of compounds. The outlet of the flow cell was connected to a split valve to divert a flow of 4 ml min^{-1} to the ESI source. Peaks were detected by positive ionization mode of MS and MS/MS detection. Mass spectrometry was carried out in the scan mode from *m/z* 50 to *m/z* 1500. ESI-MS conditions were as follows: drying gas temperature, 350 °C, drying gas flow, 10.0 l min^{-1}, nebulizing gas pressure, 0.4 Bar, fragmentation voltage, 100 V, and capillary voltage, 4500 V.

3. Results

3.1. Effect of initial dye concentration and carbon sources

Four different carbon sources and six different dye concentrations were initially tested and the results were presented in Fig. 1A and 1B.

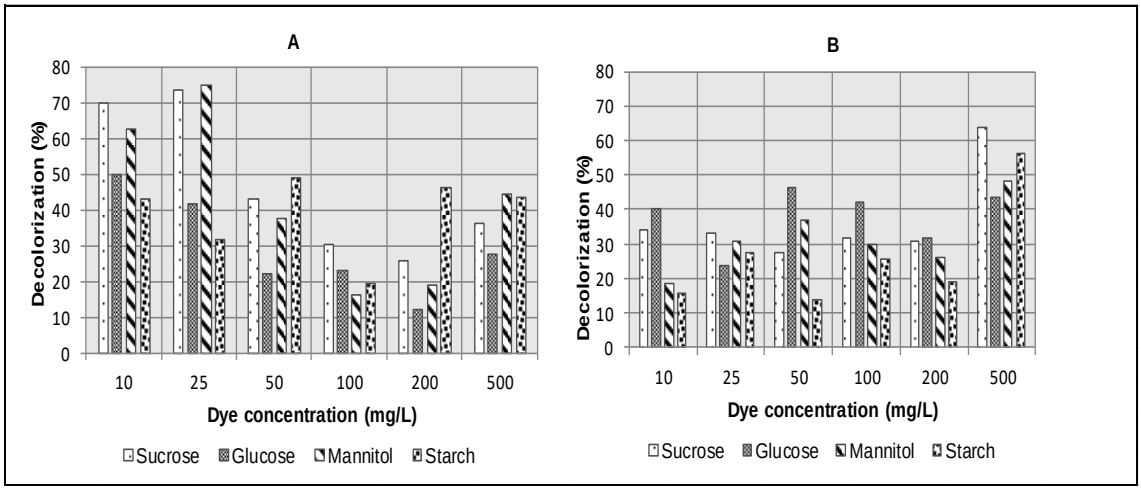

Figure 1. Maximum decolourization rates of textile dyes by *Bacillus* sp. DT-9. A: Effect of dye concentrations and carbon sources on decolourization of RV-5R. B: Effect of dye concentrations and carbon sources on decolourization of RBO-3R

While RBO-3R was decolourised by DT9 in high concentrations, the best dye removal was found in low concentrations of RV-5R dye. For RV-5R, the maximum yields were 75% in mannitol (25 mg l^{-1}), 73.33% in sucrose (25 mg l^{-1}), 50% in glucose (10 mg l^{-1}), and 48.89% in starch (25 mg l^{-1}). The best decolourization rates were 63.64% in sucrose, 56.30% in starch and 48.04% in mannitol at 500 mg l^{-1} RBO-3R. In other words, maximum decolourization was determined in sucrose and mannitol at 25 mg l^{-1} RV-5R and sucrose at 500 mg l^{-1} RBO-3R. There was no different significantly between mannitol and sucrose for decolourization of RV-5R. Also, it was found that sucrose was the common for both of dyes. Because of this, we are preferred sucrose in other steps of our study.

3.2. Effect of pH and temperature

In generally, the dye removal was above 50% at all pH levels tested in sucrose and mannitol for RV-5R. In RBO-3R, while it was shown that the DT9 strain had the lowest decolourization at pH 5.5, the rate of decolourization increased with an increase in alkalinity. Consequently, the best dye removal was obtained at pH 7.5 (91.66%) for RV-5R and pH 9.0 (63.64%) for RBO-3R (Fig. 2A). While the maximum colour removal of RV-5R was obtained at 37°C (91.66%), the best colour removal of RBO-3R was occurred at 30°C (64.71%) (Fig. 2B).

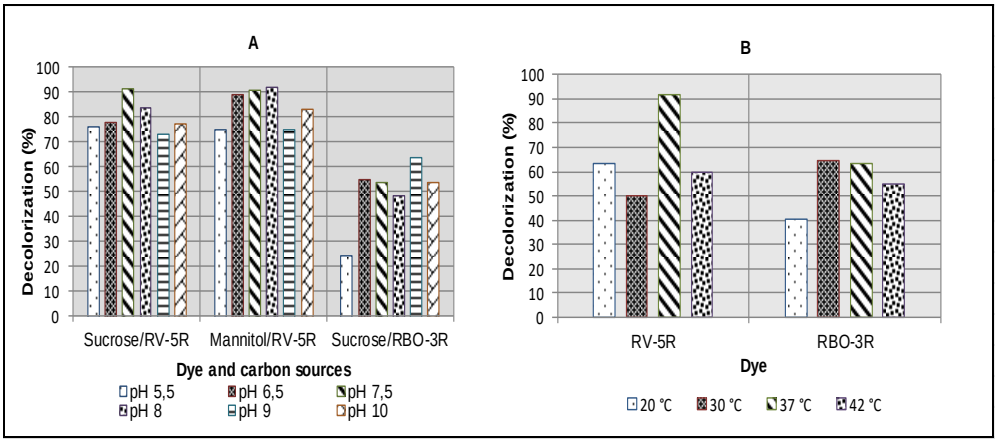

Figure 2. Maximum decolourization rates of textile dyes by *Bacillus* sp. DT-9. A: Effect of different pH levels on decolourization of RV-5R (25 mg l^{-1}) and RBO-3R (500 mg l^{-1}). B: Effect of different temperature on decolourization of RV-5R (25 mg l^{-1}, pH 7.5) and RBO-3R (500 mg l^{-1}, pH 9.0).

3.3. Effect of nitrogen sources

Different amount of ammonium chloride, peptone and yeast extract were used (Table 1). Generally, it was observed that the colour removal ratio of RBO-3R was high in yeast extract and peptone.

Table 1. Decolourization of dyes (%) by *Bacillus* sp. DT9 in different nitrogen sources.

Nitrogen sources	g l^{-1}	RV-5R	RBO-3R
Ammonium chloride	0.5	70.59	88.1
	1.0	86.66	63.27
Peptone	0.5	60.0	96.61
	1.0	31.25	98.41
Yeast extract	0.5	35.29	92.86
	1.0	58.88	24.24

In contrast to yeast extract and peptone, amonium chloride affected positivelly RV-5R decolourization. While, the maximum colour removal ratio of RV-5R was 86.66% in medium with 1 g l^{-1} ammonium chloride, the maximum decolourization ratio of RBO-3R was 98.41% in medium with 1 g l^{-1} peptone.

3.4. Immobilization

The decolourization rates of RBO-3R by immobilized cells in Ca-alginate were observed to be same (30%). The maximum dye removal rate was 92.31% for 1.0 g of cell and 86.67% for 0.5 g cell in concentration of 25 mg l^{-1} RV-5R (Table 2).

Table 2. Decolourization of RV-5R and RBO-3R by immobilized cells in Ca-alginate.

Strain	Cell pellet (g)	RV-5R (25 mg l^{-1})	RBO-3R (500 mg l^{-1})
DT-9	0.5	86.67	30.0
	1	92.31	30.0

3.5. Enzyme activity

The result of plate assay was found as negative and this showed that the enzyme activity of DT9 was not extracellular, it could be intracellular. Therefore the cell-free extract was prepared and used as enzyme source for determination of laccase activity. The enzyme activity range of DT9 was 4.86-8.48 nmol ml^{-1} (Table 3).

Table 3. Activity of laccase in *Bacillus* sp. DT9

Bacterium	Medium	Activity (nmol ml^{-1})
DT9	A	4.86±1.25
	B	8.48±0.69

A: 1 g l^{-1} sucrose, 1 g l^{-1} ammonium chloride, 37°C, pH 7.5, 25 mg l^{-1} RV-5R, B: 1 g l^{-1} sucrose, 1 g l^{-1} peptone, 30°C, pH 9.0, 500 mg l^{-1} RBO-3R

3.6. ESI-TOF/MS analysis of dyes and samples

In analysis of ESI-TOF-MS spectrum of pure RV-5R, the protonated molecular ion peak m/z 758.3 [M]+ Na (735+23=758) was observed (Fig. 3A). This result verified RV-5R molecular weight. After biodegradation of RV-5R in medium with sucrose (C12H22O11)/yeast extract, the result of ESI-TOF-MS system was shown that -C2H4-SO4-Na, -SO2 and two –SO3-Na groups' snapped and then molecule was converted dimeric structure (Fig.3B). Repeated peaks (74 Da and 16 Da) were appeared in sample spectrum too. Molecular ion peak of dimeric structure m/z 1054+Na and m/z 1077 was come into view in the same Figure 3B.

Similarly, pure RBO-3R was analysed by ESI-TOF-MS (Fig. 4A) and it was shown that this dye's molecular ion peak m/z 656 peak [M]+K (617+39=656) occurred. While 16 Da peaks repeated, structure of molecule indicated to be positive ionization. After biodegradation of RBO-3R in medium with sucrose (C12H22O11)/peptone, as all data's examined, the

similar repeated peaks (74 Da and 16 Da) were appeared in sample spectrum. According to this result, structure made coordination bonds with potassium or sodium and the molecule occurred was converted dimeric structure with snapped –Na (m/z 1227 (2(M-Na)+K) (Fig. 4B). Spectrum of medium with sucrose (C12H22O11)/ammonium chloride was also investigated; repeated peaks at 74 Da were shown that structure ionized making coordination bonds with potassium or sodium. Dimeric structure consisted with snapped –Na (m/z 1211 (2(M-Na) +Na). This situation was presented in Fig. 3C. Both the 74 Da and 16 Da peaks repeated were not observed in this medium. This result was demonstrated that structure could made coordination bonds ([M+H]+) with hydrogen atom. Dimeric structure can be consisted with snapped two –Na ((M-2Na) + H). This peak intensity was very low in spectrum (Fig. 4C).

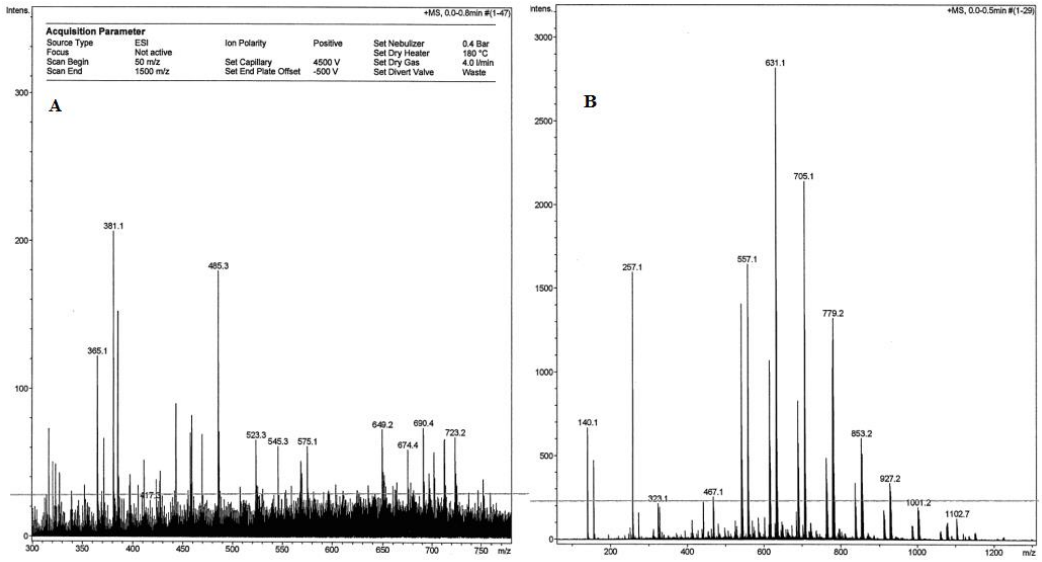

Figure 3. The spectrums of ESI-TOF-MS. A: Pure RV-5R. B: After biodegradation of RV-5R in sucrose/yeast extract medium

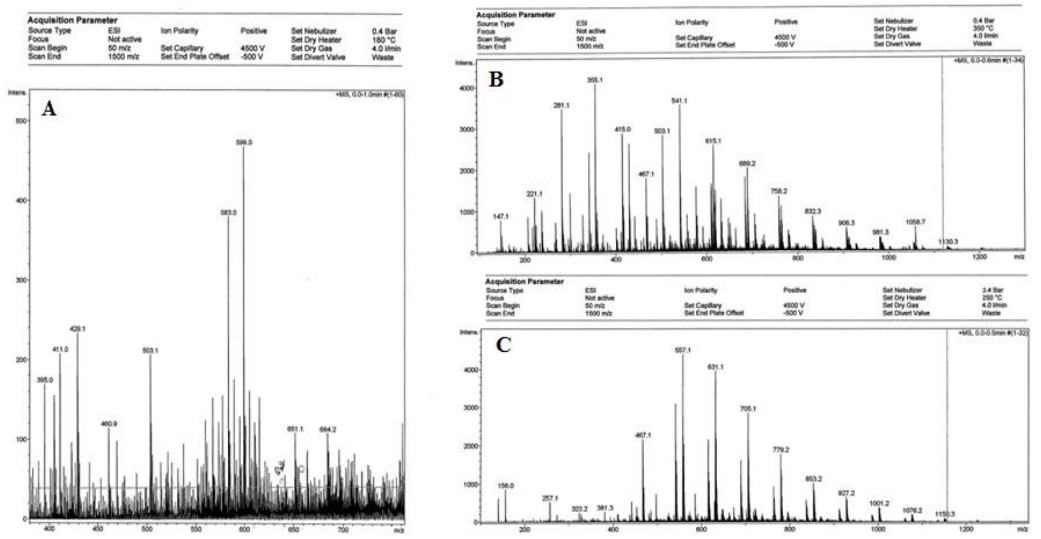

Figure 4. The spectrums of ESI-TOF-MS. A: Pure RBO-3R. B: After biodegradation of RBO-3R in sucrose/peptone medium. C: After biodegradation of RBO-3R in sucrose/ammonium chloride medium

4. Discussion

4.1. Decolourization of dyes in tested conditions

Optimum dye concentration is different for each organism. Some organisms can tolerate the high concentration of dye. Also, some organisms can die the low concentration of dye. Thus, in the first stage of our work, different dye concentrations were tested in medium with sucrose, glucose, mannitol and starch. We observed that decolourization by DT9 strain was significantly affected from dye concentrations and variety of carbon sources. While decolourization of RV-5R decreased with an increase in dye concentration, decolourization of RBO-3R increased with an increase in dye concentration. It was surprised that the maximum dye removal was achieved at high concentration of RBO-3R. Probably, the decolourization ability of *Bacillus* sp. DT9 was activated in high concentration of RBO-3R. In other words, we considered

that the enzyme that makes RBO-3R decolourization might be effective at higher concentration. It was claimed that *Pseudomonas* sp. LBC1 decolourized Direct Brown MR at a final concentration to 1000 mg l^{-1} in previous research (Telke *et al.,* 2012). Decolourization ratio increased with addition sucrose and mannitol compared to starch and glucose. Among carbon sources, sucrose affected positively to decolourization of both RV-5R and RBO-3R. In our previous study, while RV-5R by *Bacillus* sp. DT16 were decolourized in medium with yeast extract and glucose, RBO-3R was decolourized in peptone and sucrose (Arar *et al*., 2014). In a similar study, increasing in decolourization of C.I. Reactive Orange 16 by *Bacillus* sp. ADR was recorded when used of sodium acetate, sodium format, sodium succinate, sodium citrate and sodium pyruvate as electron donor (Pearce *et al*., 2003). Also, Modi *et al*., (2010) showed that maltose, sucrose, glucose and glycogen increased the dye removal activity of *B. cereus* M1. Soluble starch was found as an efficient substrate for decolourization of Azure B by *Bacillus* sp. MZS10 (Li *et al*., 2014). Similarly, Gurulakshmi *et al*., (2008) reported that the maximum rate of decolourization of Acidblue 113 by *Bacillus subtilis* was observed (90%) when starch & peptone was supplemented in the medium. Contrary to Li *et al*. (2014) and Gurulakshmi *et al*., (2008), starch was not efficient substrate for decolourization of RV-5R and RBO-3R by *Bacillus* sp. DT9 in our work. The pH is another important factor for decolourization and the optimum pH values for the maximum decolourization are usually between 6.0 and 10.0 (Chen *et al*., 2003; Kilic *et al*., 2007; Guo *et al*., 2007). In our study, optimum pH of decolourization of RV-5R was 7.5. This result is in good agreement with the observations of Anjaneya *et al*., (2011), Wang *et al*., (2009) and Gurulakshmi *et al*., (2008). For example, Anjaneya *et al*., (2011) reported that Metanil Yellow degradation by *Bacillus* sp. AK1 and *Lysinibacillus* sp. AK2 was showed at pH 7.2. Similarly, Gurulakshmi *et al*., (2008) indicated that the optimum dye decolorizing activity of *Bacillus subtilis* was occurred at pH 7.0. As indicated by Wang *et al*., (2009), the maximum decolourization of Reactive Red 180 by *Citrobacter* sp. CK3 was reached at pH 7.0 in anaerobic conditions. In our previous study, decolourization of RV-5R and RBO-3R by *Bacillus* sp. DT16 was held at pH 10.0 (Arar *et al*., 2014). Also, in present our study, the optimum pH value of decolourization of RBO-3R by *Bacillus* sp. DT9 was 9.0. On the other hand, decolourization of RBO-3R was achieved in alkali conditions unlike RV-5R. The results indicated that the decolourization pH conditions depend on the chemical structure and type of the dye. Decolourization of dyes at various temperatures value by the *Bacillus* sp. DT9 was determined (Fig. 1D). It was observed that the decolourization rates of both RV-5R and RBO-3R were more than 50% in almost all tested temperature. Maximum decolourization ratio was noticed at 37 °C (91.66%) for RV-5R and at 30ºC (64.71%) for RBO-3R. The decolourization percentage of dyes was decreased at 42 °C. Ammonium chloride, bacteriological peptone and yeast extract was used as nitrogen sources and added as 0.5 and 1.0 g l^{-1} concentrations to growth medium containing 1 g l^{-1} sucrose (the best carbon source). Although the addition of nitrogen sources in our study negatively affected on decolourization of RV-5R, they positively affected on decolourization of RBO-3R (Table 1). Especially, peptone increased the decolourization of RBO-3R from 64.71% to 96.61% (0.5 g peptone) and 98.41% (1.0 g peptone). Almost all of the RBO-3R (500 mg l^{-1}) was removed in sucrose/peptone (1:1) at pH 9.0 and 30 °C. Immobilization preserves the cells from environmental stress conditions such as pH, or exposure to toxic chemical substances and tends to have a higher level of enzyme activity than suspension cultures (Vassilev and Vassileva, 1992; Couto, 2009). Therefore, we compared the free cell activity with immobilized cell (Table 2). Consequently, when the decolourization of RBO-3R with immobilized cell was compared with that of RV-5R, the positive effect was observed for RV-5R. As indicated in Table 3, laccase activity in strain DT9 was not high level. Several decolourization enzymes such as lignin peroxidase, laccase, azoreducatase and biotransformation enzymes are found in bacteria. Literature revealed that unless strong promoter(s) or inducer(s) are present, not many strains could effectively express laccase in significant levels (Olukanni *et al*., 2010; Telke *et al*., 2011, 2012). That is, electron transfer for dye decolourization is possibly not associated with laccase as the mediator enzyme. Cytoplasmic NADH dehydrogenase or/and riboflavin reductase seemed to be candidate enzyme(s) dealing with electron transfer for decolourization.

4.2. Analysis of ESI-TOF-MS of metabolites in bio-decolourization process

To best of our knowledge, this is the first study about the ESI-TOF-MS analysis of textile dye (RBO-3R and RV-5R) and their metabolites. ESI-TOF-MS results demonstrated the bio-transformation of two dyes in our study. Moreover, each dye was dimerized to big molecule by *Bacillus* sp. DT9 laccase enzyme. There are many papers about the analysis of LC-MS (ESI mode) of textile dyes in the literature. Bansal and Sud (2013) reported photo-degradation of Reactive Red 35's metabolites and identified six products by LC-MS system. They indicated that the final metabolite was phthalic acid (Bansal and Sud, 2013). Similarly, methylene blue (MB) was analyzed by electrospray ionization mass spectrometry (ESI-MS) during the reactive adsorption to bentonite clay. Different methylene blue products were indicated in degradation process and ESI-MS analysis was verified MB molecule oxidation (Gupta *et al.,* 2015).

5. Conclusions

Our experiments indicated that *Bacillus* sp. DT9 was able to decolorize RV-5R and RBO-3R under tested conditions, but the initial concentration and other factors such as pH, temperature, carbon and nitrogen sources and types of dye influenced the behaviour of dye reduction by DT9 strain. It was observed that *Bacillus* sp. DT9 removed RBO-3R at the high test concentration. ESI-TOF-MS results were verified that both dye molecules were bio-transformed and dimerization appeared during the decolourization of dyes. These results showed that the final metabolites formed

after decolourization may be degraded by consortium of organisms.

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