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Exploring the ability of *Sphingobacterium* sp. ATM to degrade textile dye Direct Blue GLL, mixture of dyes and textile effluent and production of polyhydroxyhexadecanoic acid using waste biomass generated after dye degradation

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ABSTRACT

The degradation of textile effluent using microorganisms has been studied extensively, but disposal of generated biomass after dye degradation is a serious problem. Among all tested microorganisms, isolated *Sphingobacterium* sp. ATM effectively decolorized (100%) the dye Direct Blue GLL (DBGLL) and simultaneously it produced (64%) polyhydroxyhexadecanoic acid (PHD). The organism decolorized DBGLL at 300 mg l^{-1} concentration within 24 h of dye addition and gave optimum production of PHD. The organism also decolorized three combinations of mixture of dyes. The organism decolorized textile effluent too when it was combined with medium. The organism produced a maximum of 66% and 61% PHD while decolorizing mixture of dyes and textile effluent respectively. Molasses was found to be more significant within all carbon sources used. The activity of polyhydroxyalkanoate (PHA) synthase was found to be higher after 24 h of addition of DBGLL. The enzymes responsible for dye degradation, viz. veratryl alcohol oxidase, laccase, DCIP (2,6-dichlorophenol-indophenol) reductase, riboflavin reductase, and azo reductase were found to be induced during decolorization process of DBGLL and mixture of dyes. There was significant reduction in chemical oxygen demand (COD) and biological oxygen demand (BOD). FTIR analysis of samples before and after decolorization of dye confirmed the biotransformation of DBGLL.

1. Introduction

Synthetic plastics are used because they are easy and cheap to make and they can last a long time. Unfortunately these same useful qualities made a huge plastic pollution problem. Urbanisation has added to plastic pollution in concentrated form in cities. To overcome plastic pollution, the use of biodegradable plastic synthesized from microorganisms is a good solution. The first report for biodegradable plastic was by Lemoigne, in *Bacillus megaterium* [1]. He reported that PHAs are the cytoplasmic inclusions of microorganisms, formed in the presence of excess carbon source and when growth is impaired or restricted by the lack of other nutrients such as nitrogen, sulphur, phosphorous, i.e. in stressed conditions by enzyme PHA synthase [2]. When supply is restored, the PHA can be degraded by intracellular depolymerase and subsequently metabolized to carbon and energy source [3].

Nature is full of different colours, but the manmade activities are disturbing these colours by disposing synthetic colours in nature. Synthetic dyes are used in many industries which include tanning industries, textile industries, leather industries, etc. Many industries dispose their effluent without any treatment or by partial physical or chemical treatments. Due to this the water pollution is a serious problem nowadays. To solve this problem, use of biological agents such as microorganisms is a good option. With the use of microorganisms, one can degrade the dye residues remained in the effluents as well as reduce some other harmful effects of effluent too. Microorganisms may be used as a whole cell [4], immobilized cells [5] or by purified enzymes [6] for the degradation of textile dyes.

The main purpose of this study was the utilization of *Sphingobacterium* sp. ATM remained after degradation of dye DBGLL for the production of PHD. The disposal of microbial cells after degradation of dye is also a major problem and causes pollution. Utilization of waste biomass for PHD production minimizes the problem.

The present study deals with use of textile dye solutions or effluent for the production of PHAs. It will solve the pollution problems caused by textile industries as well as disposal of plastic in envi-

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ronment. The *Sphingobacterium* sp. ATM produces PHD, a type of mcl-PHAs [7]. In present study, *Sphingobacterium* sp. ATM degrades dye Blue GLL as well as textile effluent and simultaneously due to stress of dyes on organism it will produce PHD.

2. Experimental

2.1. Materials

All textile dyes and effluent were collected from Manpasand textile industry, located near Ichalkaranji, India. DL-3-Hydroxybutyryl CoA, DAB (3′3′-diaminobenzidine tetra hydrochloride), and riboflavin were purchased from Sigma Chemical Company (St. Louis, MO, USA). Tartaric acid was obtained from BDH Chemicals (Mumbai, India). Veratryl alcohol and other fine chemicals were purchased from Sisco Research Laboratory (SRL), India. All chemicals used were of the highest purity available and of an analytical grade.

2.2. Microorganisms and culture conditions

To study the dye degradation and PHD production we have tested totally seven microorganisms including, *Escherichia coli, B. megaterium* NCIM 2475, *Bacillus odysseyi* SUK3, *Bacillus* sp. VUS, *Bacillus thuringiensis* NCIM 2159, *Sphingobacterium* sp. ATM, and *Pseudomonas desmolyticum* NCIM 2112. *B. megaterium* NCIM 2475, *B. thuringiensis* NCIM 2159, *P. desmolyticum* NCIM 2112 are obtained from National Collection of Industrial Microorganisms, Pune, India. Other microorganisms were isolated in our laboratory. *Bacillus* sp. VUS was reported for dye degradation [8] and, *B. odysseyi* SUK3 was reported in consortium with other organisms for dye degradation [9]. All microorganisms are maintained on the nutrient agar slants having composition ($g\,l^{-1}$) peptone 10.0, yeast extract 3.0, NaCl 5.0.

2.3. Exploring the ability of Sphingobacterium sp. ATM to produce PHD while decolorizing textile dyes

All the six organisms were tested for the ability to produce of PHAs while degrading the textile dye Direct Blue GLL. The time required for production of PHAs and/or dye degradation was calculated. Also the amount of PHAs produced by organisms was calculated.

2.4. Decolorization experiment

A loopfull of microbial culture was inoculated in 250 ml Erlenmeyer flask containing 100 ml nutrient broth and incubated at 30 °C for 24 h under static condition. After 24 h of incubation, dye was added in flasks at a concentration of 500 $mg\,l^{-1}$. Five ml sample was withdrawn at different time intervals, centrifuged at 2850 × *g* for 15 min. The clear supernatant was used to measure the decolorization at the absorbance maxima of the dye, i.e. 570 nm. Un-inoculated control was used to compare colour loss during the experiment. Decolorization experiments were performed in three sets, and then percentage decolorization was calculated [10].

After decolorization of DBGLL the cells from the broth was separated by centrifugation at 2850 × *g* for 15 min and checked for PHAs production. The 24 h microbial growth in basal medium, with molasses as a carbon source was used to study the effect of static and shaking (120 rpm) condition, various temperatures (30–60 °C at pH 7.0) and pH (2.0–14.0 at 30 °C) on the decolorization of DBGLL (30 $mg\,l^{-1}$). Decolorization at increasing concentrations of DBGLL (0.05–0.5 $g\,l^{-1}$) was studied at 30 °C and pH 7.0.

2.5. Extraction of PHAs from cells

After 48 h incubation at 30 °C, 10 ml of culture was centrifuged at 8000 × *g* for 15 min. The pellet was treated with 10 ml of sodium hypochlorite and the mixture was incubated at 30 °C for 2 h. After incubation, the mixture was centrifuged at 5000 × *g* for 15 min and then washed with distilled water, acetone, and methanol respectively. After washing, the pellet was dissolved in 5 ml of boiling chloroform and was evaporated by pouring the solution on glass Petri plate and kept at 4 °C [11]. The powder was collected for further analysis after evaporation.

2.6. Preparation of cell free extract

The microorganism was grown in nutrient broth at 30 °C for 24 h. Cells were collected by centrifugation at 5600 × *g* for 20 min and suspended (100 $mg\,l^{-1}$) in 50 mM potassium phosphate buffer (pH 7.4) for sonication (sonics-vibracell ultrasonic processor), keeping sonifier output at 60 amp, giving 8 strokes, each of 30 S with 2 min interval. The temperature was maintained below 4 °C. This extract was used as source of enzyme. Same procedure was done for samples obtained after degradation of DBGLL and mixture of dyes.

2.7. Enzyme analysis

2.7.1. PHA synthase

PHA synthase activity was analyzed spectrophotometrically by using our earlier report [7]. The reaction mixture contained 1.6 mM DL-3-hydroxybutyryl CoA, 0.5 mM 5,5-dithiobis (2-nitrobenzoic acid) [DTNB] in Tris-glycerol buffer. The reaction was started by addition of enzyme. The optical density at 412 nm of thiobenzoate anion resulting from the reaction of CoA and DTNB was measured for 10 min. PHA synthase activity was analyzed at 36, 48, and 60 h after inoculation by repeated addition of DBGLL at 48 h.

2.7.2. Oxidative enzymes during decolorization

Activities of dye degrading enzymes such as veratryl alcohol oxidase and laccase were assayed spectrophotometrically in cell free extract and culture supernatant. Veratryl alcohol oxidase activity was determined by using veratryl alcohol as a substrate. The reaction mixture contained 1 mM veratryl alcohol, in 0.05 M citrate phosphate buffer, pH 3, and 0.2 ml enzyme. Total volume of 2 ml was used for the determination of oxidase activity. Oxidation of the substrate at room temperature was monitored by an absorbance increase at 310 nm due to the formation of veratraldehyde [12]. Laccase activity was determined in a reaction mixture of 2 ml containing 5 mM 3′,3′-diaminobenzidine tetrahydrate (DAB) in 0.1 M acetate buffer (pH 4.8) and increase in optical density at 410 nm [13] was measured.

2.7.3. Reductase enzymes during decolorization

Riboflavin reductase NAD(P)H:Flavin oxidoreductase was measured by monitoring the decrease in absorbance at 340 nm. Cell free extract was added to a solution (final volume 2 ml) containing 100 μM of Tris–HCl (pH 7.5), 25 μM of NADPH and 0.003 Ul^{-1} of riboflavin. Reaction rates were calculated by using a molar extinction coefficient of 6.3 $mol\,l^{-1}\,cm^{-1}$ [14]. NADH-dichlorophenol indophenol (NADH-DCIP) reductase activity was determined using a procedure reported earlier by Salokhe and Govindwar [15]. DCIP reduction was monitored at 620 nm and calculated using an extinction coefficient 19 $mM\,cm^{-1}$. The reaction mixture (5.0 ml) prepared contains 50 mM substrate (DCIP) in the 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml enzyme. From this, 2.0 ml reaction mixture was assayed at 620 nm by addition of 50 mM NADH. All enzyme assays were carried out at room temperature; reference blanks contained all components except the

Table 1
Exploring the ability of *Sphingobacterium* sp. ATM for production of PHD while degrading dye DBGLL.

Name of microorganisms	Concentration of dye used ($g\,l^{-1}$)	% Decolorization of DBGLL in 24 h	% PHAs production
E. coli	0.05	00	00
Bacillus megaterium NCIM 2475	0.05	00	58
Bacillus sp. VUS	0.10	94	00
Bacillus thuringiensis NCIM 2159	0.05	00	60
Bacillus odysseyi SUK3	0.05	82	61
Pseudomonas desmolyticum NCIM 2112	0.05	86	52
Sphingobacterium sp. ATM	0.30	100	64

enzyme. In case of azo reductase assay, the assay mixture contained 100 μM NADH and 4.45 μM Methyl red in a total volume of 2.0 ml. The volume was adjusted by using 50 mM potassium phosphate buffer [16]. One unit of enzyme activity was defined as a change in absorbance unit per ml of enzyme. MR reduction was calculated using the extinction coefficient 23.36 mM cm^{-1}.

2.8. Decolorization of mixture of dyes and textile effluent at the time of production of PHD

The *Sphingobacterium* sp. ATM was tested to degrade mixture of dyes and textile effluent. To study the mixture of dyes degradation we have tested three type of combinations of mixtures containing 5 dyes (mixture 1), 7 dyes (mixture 2), and 10 dyes (mixture 3). The mixture 1 contains dyes as Navy Blue Rx, Navy Blue 3G, Blue 2RNL, Golden Yellow HER, and Green HE4B. The mixture 2 contains Red HE8B, Red M5B, Remazol Red, Orange HE2R, Golden Yellow HER, Green HE4B, and Direct Blue GLL, while the mixture 3 contains Orange HE2R, Golden Yellow HER, Green HE4B, Navy Blue Rx, Navy Blue 3G, Blue 2RNL, Direct Blue GLL, Red HE8B, Red M5B, Remazol Red. 0.5 $g\,l^{-1}$ concentration was used for all mixtures for decolorization.

In case of textile effluent combinations with nutrient broth and basal medium with molasses was done separately. For the effluent also we have done three combinations including 30 ml effluent with 65 ml medium, 50 ml effluent with 45 ml medium and lastly 70 ml effluent with 25 ml medium. The flasks was inoculated with 5% of 24-h grown culture of *Sphingobacterium* sp. ATM and kept at 30 °C up to time of degradation.

The decolorization of mixture of dyes and textile effluent was calculated using American Dye Manufacturers' Institute (ADMI 3WL) tristimulus filter [17]. This method is applicable to coloured waters and wastewaters having colour characteristic. ADMI removal percent (%) is the ratio between the removal ADMI value at any contact time and the ADMI value at initial concentration was calculated [18] as follows:

$$\text{ADMI removal ratio}\,(\%) = \frac{\text{Initial ADMI}_{(0\,\text{h})} - \text{Observed ADMI}_{(t)}}{\text{Initial ADMI}_{(0\,\text{h})}} \times 100\%$$

where $\text{ADMI}_{(0\,\text{h})}$ and $\text{ADMI}_{(t)}$ are the initial ADMI value (at 0 h) and the ADMI value after a particular reaction time (t), respectively.

The production of PHD was checked after decolorization of mixture of dyes and textile effluent for each combination.

2.9. Characterization of textile effluent

The textile effluent and mixture of dyes was characterized by BOD and COD [18] before and after dye degradation. The textile effluent was also characterized by some other environmental parameters like hardness, alkalinity, total solids (TS), total dissolved solids (TDS), and total suspended solids (TSS) [19].

2.10. Decolorization and biodegradation studies

Decolorization of dye was monitored using UV–vis spectrophotometer (Hitachi U 2800). After complete decolorization, the decolorized medium was centrifuged at 2850 × g for 20 min. The supernatant obtained was used to extract metabolites with an equal volume of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volume of HPLC grade methanol and used for FTIR and GC–MS analysis. High performance liquid chromatography (HPLC) analysis was carried out (waters model no. 2690) on C_{18} column (symmetry, 4.6 × 250 mm) by using methanol as a mobile phase with flow rate of 1 ml min^{-1} for 10 min and UV detector at 254 nm and at 570 nm. A sample of 10 μl was injected in the system.

The Fourier transform infrared spectroscopy (FTIR) analysis of extracted metabolites was done on Perkin Elmer, Spectrum one instrument and compared with control dye in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio 5:95, pellets were fixed in sample holder, and the analysis was carried out. The GC–MS analysis of metabolites were carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280 °C with oven conditions as: 80 °C kept constant for 2 min increased up to 200 °C with 10 °C min^{-1} raised up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the NIST library.

2.11. Effect of various carbon and nitrogen sources

Sphingobacterium sp. ATM cells were cultivated at 30 °C for 48 h in the basal medium supplemented with glycerol, glucose, starch, molasses, fried oil as different sources of carbon and cheese whey and urea as a nitrogen source (1%) to check the effective carbon source for both dye degradation and PHA production and to confirm production of PHAs in depletion of nitrogen. Effect of substrate concentration was studied by adding 1%, 2%, 3% and 4% molasses to the basal medium.

2.12. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons. Readings were considered significant when P was ≤0.05.

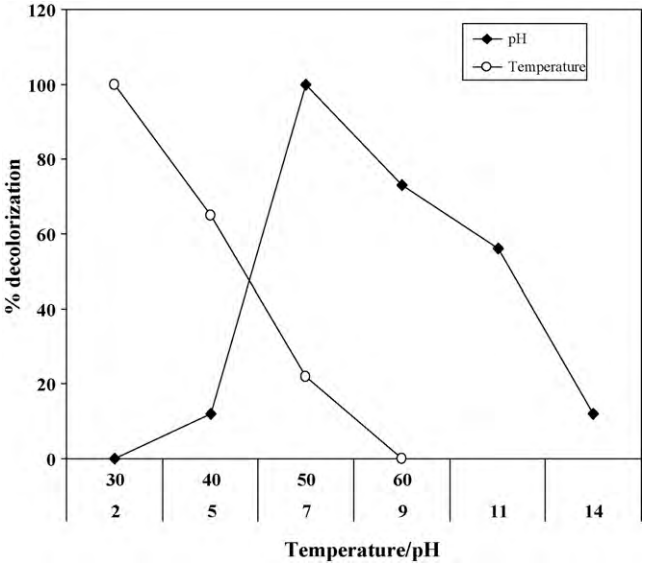

Fig. 1. Effect of different temperature and pH on decolorization of DBGLL.

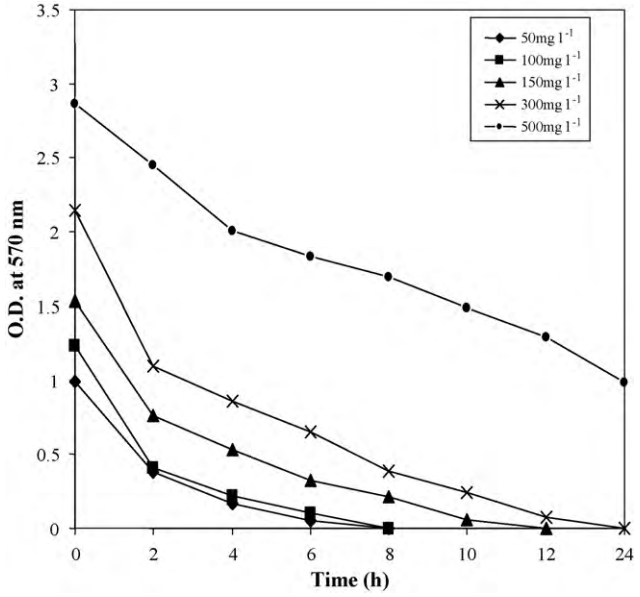

Fig. 2. % Decolorization of different DBGLL concentration observed at 570 nm.

3. Results and discussion

3.1. Exploring the ability of Sphingobacterium sp. ATM to produce PHD while decolorizing textile dyes

Many microorganisms degrade dyes [20,21] and produce PHAs [22] separately. We checked the ability of microorganisms to produce PHAs while degrading the dye DBGLL. We observed that *E. coli* does not produce PHAs and degrade the dye. In case of *B. megaterium* NCIM 2475, *B. thuringiensis* NCIM 2159 we observed only production of PHAs and reverse in case of *Bacillus* sp. VUS which shows only dye degradation. *P. desmolyticum* NCIM 2112, *Sphingobacterium* sp. ATM showed both production of PHAs and the dye degradation at the time of PHA production (Table 1). But the concentration of PHA production was found to be high for *Sphingobacterium* sp. ATM and also highest concentration of dye was found to be degraded. *Sphingobacterium* sp. ATM was reported for production of polyhydroxyhexadecanoic acid in our earlier report [7].

As *Sphingobacterium* sp. ATM was found to be effective among all screened microorganisms it was used for all our future work.

3.2. Decolorization experiment

Experiments for dye degradation and PHD production were carried out initially at both static and shaking conditions. But the degradation of dye was found to be inhibited in case of shaking condition and PHD production remained constant in both cases. Khalid et al. [23] reported that the shaking condition delays the degradation of textile dyes which supports our observation. As only static conditions gave degradation all further experiments were carried out at static conditions. The optimum temperature for dye decolorization was found to be 37 °C, the rate of decolorization decreases with increase in temperature (Fig. 1). *Sphingobacterium* sp. ATM showed decolorization most prominently at neutral pH as well as it decolorized DBGLL in alkaline pH range too (Fig. 1).

Different dye concentrations were studied for the effect of rate of degradation of the dye DBGLL. We observed that, the time required for degradation increased with increasing concentrations of the dye. The PHA production was found to be optimum at 24 h of dye addition at which the highest dye concentration that was completely degraded by our isolated microorganism was 300 mg l^{-1} (Fig. 2). Thus, this dye concentration was selected for further studies.

3.3. Enzyme analysis

3.3.1. PHA synthase

The reports showed that the activity of PHA synthase increases when there was an unfavourable condition for the growth [24]. *Sphingobacterium* sp. ATM showed increase in activity when repeated addition of dye was done (Table 2). When compared with the activity without repeated dye addition it was observed that the activity at 60 h in case of repeated dye addition was higher than that obtained without dye repetition, also as activity of PHA synthase

Table 2
Production of PHD and activity of PHA synthase at different time intervals in the presence and absence of DBGLL.

		Incubation time after inoculation (h)		
		36	48	60
PHA synthase activity (U/h/mg protein)	Without dye repetation at 48 h	0.18 ± 0.0003	0.39 ± 0.0005	0.19 ± 0.0001
	With DBGLL repetation at 48 h	0.22 ± 0.0012*	0.44 ± 0.0018*	0.59 ± 0.0123**
PHD production (% dry weight)	Without dye repetation at 48 h	26	58	43
	With DBGLL repetation at 48 h	35	64	65
PHAs yield (g l^{-1})	Without dye repetation at 48 h	0.923	3.48	1.40
	Without dye repetation at 48 h	0.923	3.48	5.05

Values are a mean of three experiments ± SEM.
Significantly different from control (0 h) at *$P < 0.05$ and **$P < 0.001$ by one-way ANOVA with Tukey-Kramer comparison test.

Table 3
Enzyme activities in *Sphingobacterium* sp. ATM at (0 h) and after 24 h of addition of DBGLL.

Enzyme	0 h	24 h			
		DBGLL	Mixture 1	Mixture 2	Mixture 3
Veratryl alcohol oxidase[a]	0.044 ± 0.003	0.439 ± 0.010*	0.051 ± 0.005*	0.075 ± 0.020*	0.034 ± 0.001*
Laccase[a]	0.024 ± 0.0013	0.511 ± 0.010*	0.030 ± 0.002*	0.041 ± 0.004*	0.043 ± 0.003*
DCIP reductase[b]	2.11 ± 0.053	10.79 ± 0.053***	6.04 ± 0.457***	7.43 ± 0.023***	7.06 ± 0.021***
Riboflavin reductase[c]	0.477 ± 0.008	6.14 ± 0.091***	1.18 ± 0. 018*	1.38 ± 0.002*	2.17 ± 0.366***
Azo reductase[d]	0.617 ± 0.040	2.82 ± 0.053***	1.66 ± 0.009***	2.03 ± 0.007***	1.41 ± 0.002***

Values are a mean of three experiments ± SEM.
Significantly different from control (0 h) at $^{*}P < 0.05$ and $^{***}P < 0.001$ by one-way ANOVA with Tukey-Kramer comparison test.
[a] Activity in U/min/mg protein.
[b] μg of DCIP reduced/min/mg protein.
[c] μg of riboflavin reduced/min/mg protein.
[d] μM of MR reduced/min/mg protein.

Table 4
Decolorization of mixture of dyes using *Sphingobacterium* sp. ATM and PHD production.

Sample	ADMI (units)		ADMI removal ratio (%)	PHD production (% dry weight)
	0 h	24 h		
Mixture 1	1507	529	64.89	59
Mixture 2	2655	720	72.88	63
Mixture 3	2394	922	61.48	66

increases the concentration of PHD found to increase (Table 2). After releasing stress of dye that is after dye degradation the activity and ultimately PHD production reduces this supports the report that after conditions are restored the PHA production reduces [25].

3.3.2. Oxidative enzymes during decolorization

Degradation of DBGLL by using enzyme lignin peroxidase was reported earlier using *Comammonas* sp. UVS [26]. In case of *Sphingobacterium* sp. ATM we observed the induction of veratryl alcohol oxidase and laccase when activities of before and after dye addition was studied which was completely different from earlier report of Jadhav et al. The purified veratryl alcohol oxidase was reported earlier for degradation of dyes [27] using *Comammonas* sp. for DBGLL but it gave different kind of products than the products we have obtained. The laccase of *P. desmolyticum* NCIM 2112 was reported for dye degradation [28].

In case of mixture of dyes also we observed induction of veratryl alcohol oxidase and laccase, revealing their role in the degradation of mixture of dyes (Table 3).

3.3.3. Reductase enzymes during decolorization

The enzymatic analysis of *Sphingobacterium* sp. ATM for reductive enzymes showed induction of DCIP reductase, riboflavin reductase (Table 3). Azo reductase was also found to induce in the presence of dye DBGLL. The earlier report confirms the use of azo reductase for degradation of dyes [29].

When compared with our earlier report [7] it was observed that in *Sphingobacterium* sp. ATM azo reductase activity increased tremendously than earlier which confirms role of azo reductase in DBGLL degradation because DBGLL contains more azo bonds than DR5B in its structure.

In case of mixture of dyes also we observed induction of DCIP reductase, riboflavin reductase, and azo reductase, revealing their role in the degradation of mixture of dyes (Table 3).

3.4. Decolorization of mixture of dyes and textile effluent at the time of production of PHD

Decolorization of mixture of dyes and textile effluent by different microorganisms was reported earlier in few reports [30,31]. While decolorizing mixture of dyes, it was observed that mixture 2 was decolorized effectively than other two mixtures at 24 h of dyes addition. It showed 72.88% ADMI removal which was found to be higher than other mixtures (Table 4). The PHD production was found to be more for mixture 3. It may be due to maximum stress of dyes because mixture 3 contains ten different textile dyes while other mixture contains less than it. The mixture 3 contains combination of all azo, direct, and vinyl sulphone dyes, so the structure variability may induce the enzyme PHA synthase which leads to maximum production of PHD.

In case of textile effluent the time required for decolorization varies with concentration of effluent in mixture of medium and effluent (Table 5). Lesser concentration of effluent gets decolorized in lesser time and combination containing more concentrated amount of effluent requires more time. The PHD production was found to be more in 50 ml effluent and 50 ml medium in both cases of medium used.

Table 5
Decolorization of industrial effluent using *Sphingobacterium* sp. ATM and PHD production.

Medium	Time required for decolorization (h)	ADMI removal (%)	BOD reduction (%)	COD reduction (%)	PHD production (%)
Effluent + NB (ml)					
30 + 65	24	81	71	62	54
50 + 45	38	78	79	66	56
70 + 25	51	61	52	58	49
Effluent + molasses medium (ml)					
30 + 65	24	83	69	64	58
50 + 45	38	79	78	68	61
70 + 25	51	67	56	59	52

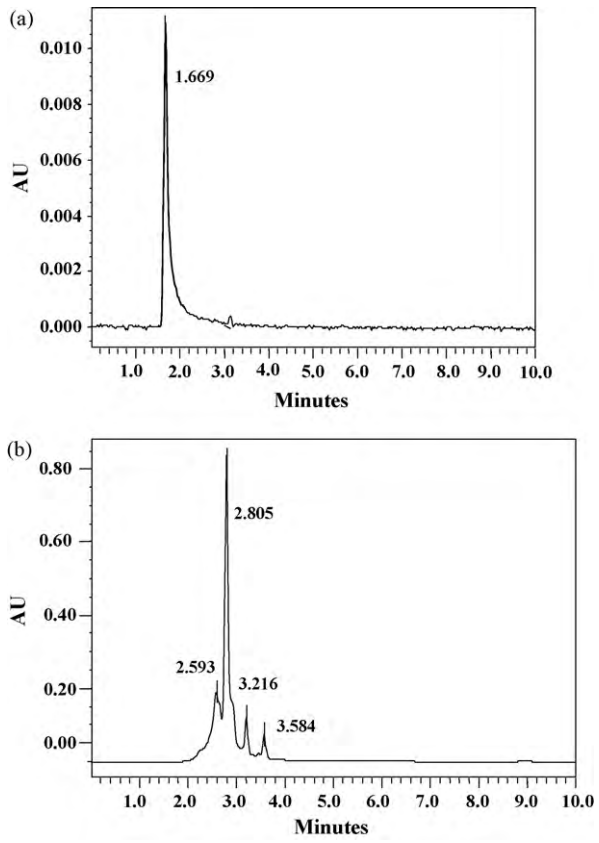

Fig. 3. (a) HPLC pattern of the dye DBGLL before degradation. (b) HPLC pattern of the metabolites obtained after degradation of DBGLL after 24 h.

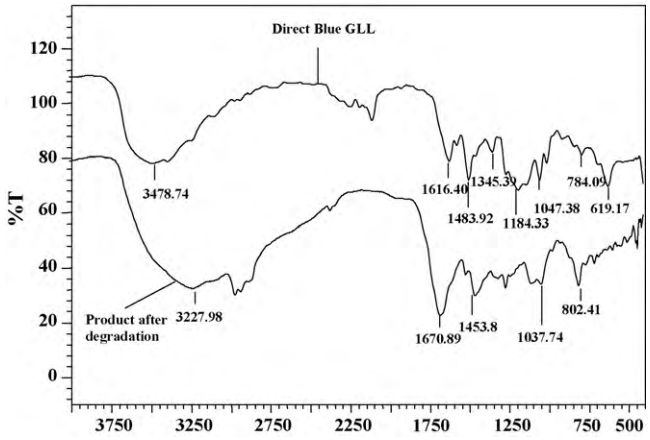

Fig. 4. FTIR spectrum of control dye DBGLL and metabolites formed after 24 h.

3.5. Characterization of textile effluent

Sukumar et al. [32] showed degradation of textile effluent using genetically improved bacteria while Satiya et al. [33] degraded effluent using white rot fungi. The textile effluent has many harmful parameters in it. It was confirmed by characterizing the effluent by environmental parameters. The BOD and COD were found to be high for untreated effluent than the effluent treated by *Sphingobacterium* sp. ATM. The BOD and COD values were found to decrease effectively for all combinations of effluent and medium (Table 5). The other parameters like alkalinity, hardness were also found high. The alkalinity of effluent was found to be 900 mg l^{-1} and the hardness was 1400 mg l^{-1}. The effluent contains total dissolved solids 3960 mg l^{-1}, total dissolved solids 360 mg l^{-1}, and total suspended solids 3600 mg l^{-1}.

3.6. Decolorization and biodegradation studies

The decolorization of DBGLL was studied earlier by *Comammonas* sp. UVS [26]. But the degradation and enzymatic pattern was found to be different when it was degraded by *Sphingobacterium* sp. ATM. When the degraded product was analysed by UV–visible spectrophotometer it was observed that complete loss of absorbance at 570 nm confirms 100% decolorization. The decrease in absorbance was due to either adsorption of dye on microbial cells or by degradation [34]. But in our case we did not observe any kind of adsorption of dye on cells so it ultimately is degradation.

The HPLC analysis of dye sample collected at 0 h incubation showed peak at 1.669 min (Fig. 3a). As the decolorization progressed, the biodegradation of parent compound was observed with four detectable peaks (retention time 2.593, 2.805, 3.216, and 3.584 min) in 24 h extracted sample, which confirms formation of four products after the degradation of DBGLL (Fig. 3b). Comparison of FTIR spectrum of control dye and the products formed after 24 h, i.e. complete degradation revealed the biodegradation of dye DBGLL by *Sphingobacterium* sp. ATM (Fig. 4). The spectrum of control dye showed the peaks at 3478.74 cm^{-1} for primary amines, free NH secondary amines with N–H stretching, peak at 1616.40 cm^{-1} represents N=N stretching as in case of azo compounds, peak at 1483.92 cm^{-1} for alkanes $-CH_2-$ with C–H deformation, peak at 1345.39 cm^{-1} for aromatic secondary amines with C–N vibration, peak at 1184.33 cm^{-1} for sulfur containing compound with S=O stretching, peak at 1047.38 cm^{-1} for sulfonic acids with S=O stretching, peak at 784.09 cm^{-1} for benzene ring with three adjacent H atoms, C–H deformation, and peak at 619.17 cm^{-1} for acyclic compound C–N starching confirms functional groups present in dye structure.

Products obtained showed the presence of peak at 3227.98 cm^{-1} for amines with N–H stretching, peak at 1670.89 cm^{-1} for alkenes with C=C stretching, peak at 1453.89 cm^{-1} for alkanes with C–H deformation, peak at 1037.74 cm^{-1} for sulfonic acid with S=O stretching, peak at 802.41 cm^{-1} for benzene ring with three adjacent H atoms, C–H deformation. Loss of peak at 1616.40 cm^{-1} in product spectra confirms loss of azo bond while degradation which supports the enzymatic pattern of *Sphingobacterium* sp. ATM. The presence of peak at 3227.98 cm^{-1} for amines confirms GC–MS analysis, i.e. formation of naphthalene-1-yl amines and naphthalene-2-yl amines.

We have proposed a pathway for degradation of DBGLL (Fig. 5) on the basis of GC–MS analysis. Veratryl alcohol oxidase plays an important role in the detoxification of various hazardous dyes by carrying out oxidative cleavage [27]. The enzyme veratryl alcohol oxidase cleaved DBGLL asymmetrically to form [A] 4-phenyl azo naphthaleneidiazine [R_t 22, 758 min, Mw (m/z), 260 (M−3)] and [B] naphthalene-1-yl-naphthale-n-2-yl-diazine [R_t 24, 992 min, Mw (m/z), 281 (M+3)]. Further degradation of [A] gave product [C] [R_t 26, 883 min, Mw (m/z), 247 (M−1)] further cleavage in product [C] yields product [D] naphthalene-1-yl amines [R_t 20, 350 min, Mw (m/z), 143 (M−2)] and one unidentified product [1]. Degradation of [B] gave products [D] naphthalene-1-yl amines and [E] naphthalene 2-yl amines with same retention time [R_t 20, 350 min, Mw (m/z), 143 (M−2)]. Formation of product [C], [D], and [E] was due to action of azo reductase. Azo dyes can be cleaved symmetrically and asymmetrically, with an active site available for an enzyme to excite the molecule [35].

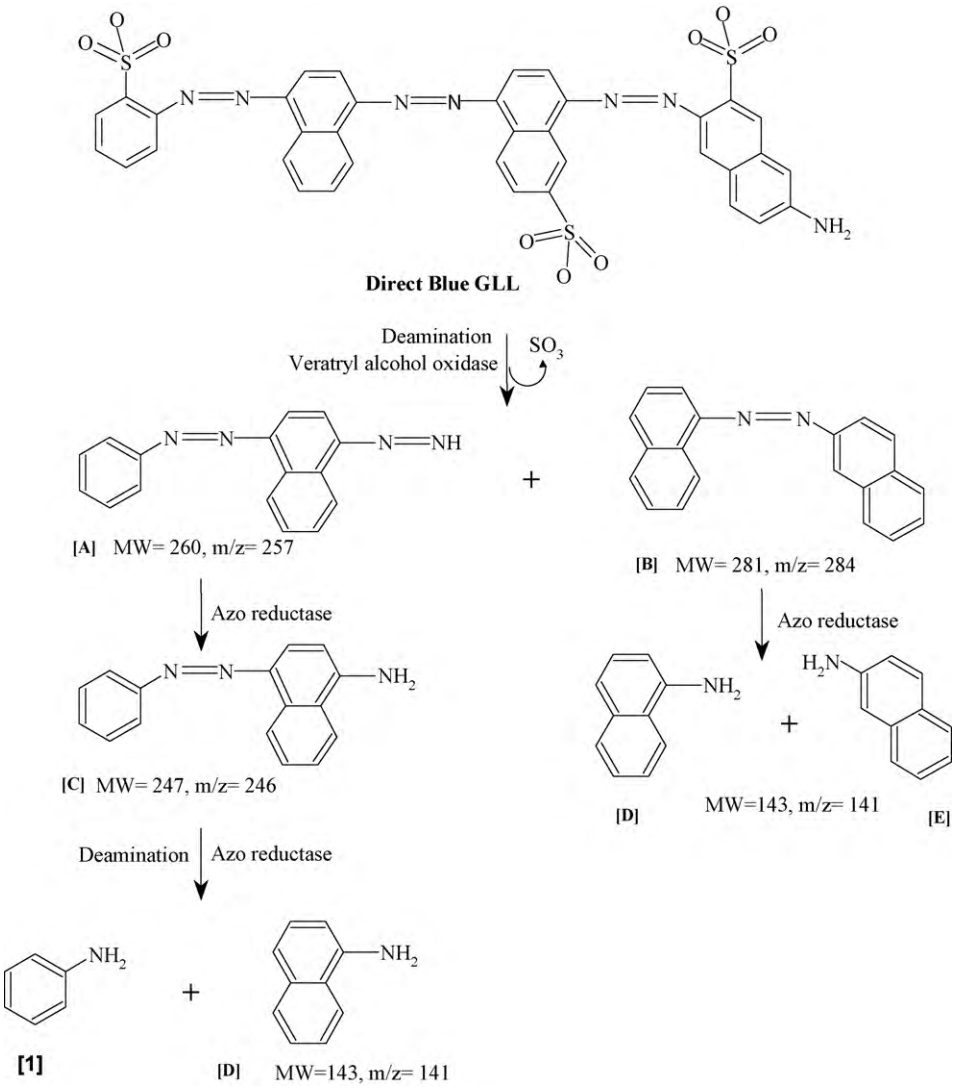

Fig. 5. Proposed pathway for the degradation of DBGLL using *Sphingobacterium* sp. ATM.

3.7. *Effect of various carbon and nitrogen sources*

Many microorganisms produce PHAs in the presence of many types of substrates which also include some industrial and agricultural wastes too [36]. *Sphingobacterium* sp. ATM was reported to produce polyhydroxyhexadecanoic acid a type of mcl-PHAs in our earlier report [7]. While degrading DBGLL, molasses was found to be more prominent substrate compared to other used (Table 6). Maximum production of PHD in the presence of molasses confirms reports that PHAs are produced when organism has high availability of carbon source [37]. Use of molasses also decreases pollution problem of sugar industries. So for all degradation studies molasses medium was used. As we increase molasses concentration up to certain limit the concentration of PHD produced increases and then remains constant and same as in case of degradation of DBGLL (Table 7).

The *Sphingobacterium* sp. ATM does not show production of PHD when the medium was supplemented with nitrogen sources like urea and in case of cheese whey was negligible. It has been reported that PHAs are produced in deficiency of nitrogen [38]. But in both cases, complete degradation of DBGLL was observed.

Table 6
Effect of carbon sources on degradation of Direct Blue GLL and production of PHD using *Sphingobacterium* sp. ATM.

Carbon source	% Decolorization of Direct Blue GLL (300 mg l^{-1}) in 24 h	PHAs production (% dry weight)
Glycerol	100	52
Glucose	100	56
Starch	100	53
Molasses	100	64
Fried oil	100	48
Cheese whey	100	08
Urea	100	00

Table 7
Effect of molasses concentration on degradation of Direct Blue GLL and production of PHD using *Sphingobacterium* sp. ATM.

Molasses concentration (%)	% Decolorization of Direct Blue GLL (300 mg l^{-1}) in 24 h	PHAs production (% dry weight)
1	100	64
2	100	66
3	84	68
4	72	68

4. Conclusion

The present study indicated the potential of *Sphingobacterium* sp. ATM to decolorize and degrade textile dye DBGLL as well as use of waste biomass remained after dye degradation for production of polyhydroxyhexadecanoic acid. The strain degrades 0.3 g l^{-1} of DBGLL within 24 h which was the optimum time for PHD production. Addition of molasses in basal medium enhances dye decolorization as well as PHD production. The presence of azo reductase and veratryl alcohol oxidase in culture supernatant indicate their role in dye degradation. Repeated addition of dye enhances PHA synthase activity confirming the stress of dye increases PHD production.

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