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# Biodecolorization of textile azo dye using *Bacillus* sp. strain CH12 isolated from alkaline lake

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ABSTRACT

Textile azo dye decolorizing bacteria were isolated from alkaline Lakes Abaya and Chamo using Reactive Red 239 (RR239) dye. Through subsequent screening process, strain CH12 was selected to investigate the effects of nutrient supplement, DO, pH, temperature, dye concentration and types on decolorization. Based on 16S rRNA gene sequence analysis, strain CH12 was identified as *Bacillus* sp. Decolorization efficiencies were significantly enhanced with carbon (≥98%) and organic nitrogen (~100%) supplements. Complete decolorization was also observed under anoxic and anaerobic conditions, and at the temperature of 30 °C and the pH of 10. However, the azo dye decolorization efficiency of strain CH12 was significantly reduced when $NaNO_3$ (1–8%) was supplemented or under aerobic culturing condition (≤6%), indicating that RR239 was less preferred electron acceptor. Overall, strain CH12 can be a promising candidate for decolorization applications due to its potential to effectively decolorize higher RR239 concentrations (50 – 250 mg/L) and six additional dyes.

## 1. Introduction

Discharge of wastewater from textile, paper, leather, food, plastic and cosmetic industries causes serious environmental pollution [1,2]. In textile industry, the main environmental concern is colored water originated from dyeing process. Currently, there are more than 100,000 different commercially available dyes at market [3], and their annual production capacity has been estimated to be over $7 \times 10^5$ tones [4]. They are chemically diverse in nature and can be divided into azo, reactive, triphenylmethane, heterocyclic and polymeric dyes [5]. Azo dyes are one of the most widely used dyes and can account for 70% of the total dye production [1]. They have one or more azo groups ($R_1$−N═N−$R_2$) and aromatic rings mostly substituted by sulfonate groups [1,5,6].

Since dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments [1,7,8]. During dyeing process, approximately 10–15% of the dye is released into wastewater stream and can cause serious environmental and health hazards [9]. Disposal of dye containing wastewater into aquatic ecosystem reduces photosynthetic activities by impeding the light penetration into deeper layers [1,3,10], which leads to the depletion of dissolved oxygen (DO) and the loss of biodiversity in the aquatic environment [4]. There are also considerable evidences that certain anaerobic metabolites of dyes are toxic, carcinogenic and mutagenic agents to microorganisms, aquatic life and human beings [2]. These highlight the need of treating textile dye containing effluent before discharging it into water bodies. The removal of color from wastewaters is often more problematic than the removal of the soluble colorless organic substances [5].

A wide range of biological, chemical and physical methods have been used to treat textile dye effluents [1,11]. Although the physical and chemical methods are technically feasible for treatment of color wastewater, they have inherent drawbacks such as high operative cost, formation of hazardous byproducts and intensive energy consumption [9,10,12]. As a viable alternative, biological treatment methods using aerobic and anaerobic microorganisms [6,11] have received increasing interest owing to their high effectiveness, lower sludge production and ecofriendly nature [4]. It has been reported that many microorganisms, such as fungi [7], algae [10] and bacteria [3,5,13] can be used for the decolorization of dye wastewater. Bhatt et al. [15] mentioned that isolation of such microorganisms has greatly contribute to dye removal in both developed and developing countries.

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Several studies have isolated and characterized dye decolorizing bacteria from textile effluent discharging sites [3,9,13–17]. For instance, Arora et al. [9] and Asad et al. [3] isolated effluent adapted microorganisms (*Bacillus firmus* and *Halomonas* sp., respectively) that had the potential of reducing textile azo dyes. Other dye decolorizing bacteria, such as *Pseudomonas aeruginosa* [15] and *Comamonas* sp. UVS [13] were also isolated from waste contaminated sites. However, only a few works were devoted to isolate and characterize microorganisms from dye-uncontaminated environment for treating textile dye contain contained effluents [18–20].

Given the characteristics of textile wastewater, the present study hypothesized that dye degrading organisms might be isolated from alkaline lake environment. Since textile industries use different salt and sodium hydroxide in wet processing steps for dye fixation, the effluents are characterized by high salinity and alkalin alkalinity (pH = 11.0–11.5) [21]. Hence, the bioremediation in such environment requires the presence of alkaliphilic and halophilic microorganisms, which are able to adapt and physiologically function under such harsh conditions. Soda lakes represent a stable alkaline environment with diverse microorganisms, which may have a potential for biotechnological applications [22,23]. Generally, alkaliphilic microorganisms have an optimal growth pH around 10. Thus, they are mainly found in extremely alkaline environment, such as Western Soda Lakes in the United States and the Rift Valley Lakes in East Africa [23].

In this study, dye degrading microorganisms were isolated from two East African Rift Valley Lakes Abaya and Chamo in Ethiopia, which were not contaminated by any industrial waste. No research has been conducted to investigate the potential of microorganisms from these lakes to treat industrial wastewater. Samples enriched in Reactive Red 239 (RR 239) dye containing mineral salt media (MSM) were used to isolate morphologically distinct colonies. The effects of nutrient supplement, culturing conditions, pH, temperature, dye concentrations and types of dye on decolorization were evaluated using the best isolate.

## 2. Materials and methods

### 2.1. Experimental setup

Batch experiment of dye decolorization was conducted in 1000 mL capacity reactor (Fig. 1). The reactor was sealed to ensure an anoxic condition. It had a tightened lid with two holes for gas removal and sampling. Gas products (particularly carbon dioxide that contributes to pH drop) from the reactor were removed using potassium hydroxide (KOH) solution. A sampling tube was inserted deep into the reactor at one side and attached to a sterile syringe on the other side. When the sample was drawn with a sterile syringe, the opening and closing of the tube was regulated with a control valve. Since the system was designed to be anoxic, the valve was immediately closed after sampling to prevent the flow of gas into the reactor.

### 2.2. Source of microbial culture

Alkaline sediment samples were collected from the Ethiopian Rift Valley Lakes of Abaya and Chamo. The rationale of using these alkaline lake inocula is that the alkaliphilic microorganisms in these lakes may be able to adapt to the alkaline environment of dye contaminated wastewater and contribute to decolorization. The triplicate average pH values of Abaya and Chamo were 8.5 ± 0.4 and 9.1 ± 0.2, respectively. Detailed physico-chemical characteristics of the lakes are given in Table 1.

### 2.3. Media composition

Mineral salt media used by Arora et al. [9] was modified by adjusting the pH to alkaline range. The composition includes (g/L): $Na_2HPO_4$ (3.6), $KH_2PO_4$ (1.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4$ (1.0), $CaCl_2$ (0.10), $FeC_6H_5O_7$ (0.01) and 10 mL/L of trace element solution. The trace element solution has the following composition (mg/L): $H_3BO_3$ (30.0), $ZnSO_4{\cdot}7H_2O$ (10.0), $Na_2MoO_4{\cdot}2H_2O$ (3.0), $MnCl_2{\cdot}4H_2O$ (3.0), $NiCl_2{\cdot}6H_2O$ (2.0), $CoCl_2{\cdot}6H_2O$ (1.0), and $CuCl_2{\cdot}2H_2O$ (1.0). Stock solutions of glucose (50%, w/v) and yeast extract (10%, w/v) were sterilized separately and added to the media to maintain final concentrations of 0.5% (w/v) and 0.01% (w/v), respectively. During MSM-agar plate preparation, 2% (w/v) agar was added to the media. An alkaline pH of the media was maintained by using separately sterilized $Na_2CO_3$ (25%, w/v).

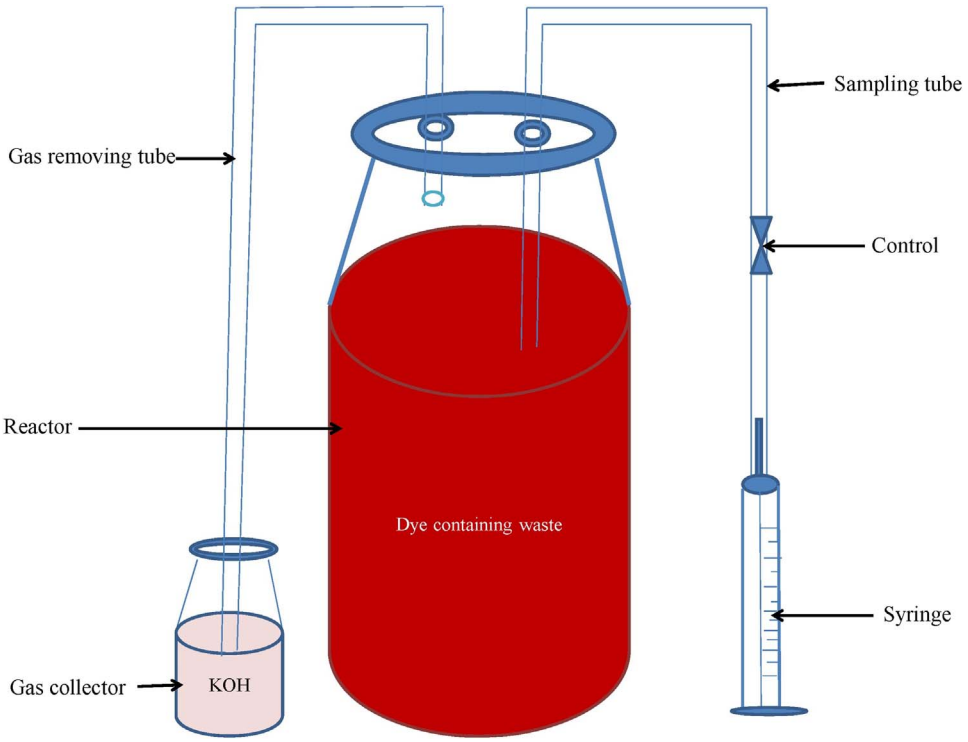


**Fig. 1.** Schematic of the decolorization reactor setup.

**Table 1**
Physico-chemical characteristics of Abaya and Chamo Lakes.

| Parameter | Abaya | Chamo |
|---|---|---|
| pH | 8.5 ± 0.4 | 9.1 ± 0.2 |
| Salinity (mg/L) | 638.0 ± 0.1 | 1154.5 ± 0.6 |
| Chloride (mg/L) | 74.2 ± 0.3 | 141.0 ± 0.1 |
| Alkalinity (as $CaCO_3$ mg/L) | 528.0 ± 1.8 | 814.0 ± 4.8 |
| Potassium (mg/L) | 12.0 − 19.80 | 20.0 − 22.50 |
| Temperature (°C) | 23.8 ± 2.4 | 25.3 ± 2.1 |
| Conductivity(ms/cm) | 1.3 ± 0.4 | 2.0 ± 0.4 |
| TDS (mg/L) | 757.0 ± 1.9 | 980.0 ± 4.6 |
| TSS (mg/L) | 248.0 ± 0.1 | 350.2 ± 0.5 |
| TS (mg/L) | 1005.2 ± 0.3 | 1330.0 ± 0.1 |
| DO (mg/L) | 4.5 ± 0.3 | 4.8 ± 0.2 |

DO = Dissolved oxygen, TDS = Total dissolved Solids, TSS = Total suspended solids, TS = Total solids.

### 2.4. Dyes

All dyes used in this study were pure reactive dyes, and were generously donated by Ayka Addis and Adei Abeba textile factory in Ethiopia. Reactive Red 239, a commonly used commercial reactive dye, was chosen for acclimatization, screening and decolorization experiments. In addition, other reactive dyes with different chemical structures, including Reactive Red 120, Reactive Red 141, Reactive Yellow 84, Reactive Yellow 160, Reactive Blue 198 and Reactive Blue 19 were used to investigate the decolorizing ability of the best isolate obtained from RR 239 experiment. All dyes used in this study contain halogen (chlorine/fluorine) and sodium sulfonate ($SO_3Na$) groups in their molecular formula. The detailed descriptions of the dyes are given in Table 2.

### 2.5. Enrichment, isolation and screening of dye degrading microorganisms

Alkaline sediment samples collected aseptically from Lakes Abaya and Chamo were enriched in azo dye containing MSM. Sterilized MSM containing 10 mg/L RR 239 was inoculated with sediment samples (10%, w/v) and incubated at ambient temperature under anoxic condition. Ten percent of samples were further transferred to fresh dye containing media within a week when constant decolorization was achieved. After each transfer, the enriched samples were serially diluted ($10^{-1}$–$10^{-7}$) and plated on MSM agar containing 10 mg/L of RR 239 and then incubated under anoxic condition at ambient temperature. Finally, 135 morphologically different colonies were isolated and further purified via spread plate method. The colonies were stored at 4 °C for immediate use. The samples were also stored at −70 °C using 15% glycerol.

Each pure isolate was tested for color removal in liquid MSM containing RR 239. A loop full of cell culture from each slant were taken and allowed to growing aerobically in 250 mL capacity of Erlenmeyer flask containing sterilized liquid MSM (100 mL) without dye. The flasks were incubated on shaker at 120 revolution per minute (rpm) at ambient temperature for 4–5 days. Then, the aerobically grown cells (10%, v/v) were cultured in the batch reactor containing liquid MSM and 10 mg/L of RR 239. The preparation were incubated at ambient temperature under anoxic condition. Decoloriation activities were monitored visually and using UV–vis spectrophotometer (UV/VIS spectrophotometer RS-295 model, India).

For further screening, seven isolates that could completely decolorize 10 mg/L of RR 239 in liquid MSM within 24 h were grown aerobically and then portion of these cultures (10%, v/v) were allowed to growing under anoxic condition in MSM containing more higher concentration of RR 239 (50–200 mg/L) to select the most effective decolorizer.

### 2.6. Identification of the best isolate

Morphological, physiological, biochemical and molecular characterizations were conducted to identify the best isolate. Genomic DNA was extracted using a freeze-thaw method modified by Moore and his colleagues [24]. 16S rDNA was amplified using polymerase chain reaction (PCR) with universal eubacteria specific primers of A8f (5′-CTGAGCCAGGATCAACTCT-3′) and H1542r (5′-TGCGGCTGGATCACCTCCTT-3′) [25].

Fifty-microliter reaction mixtures were prepared by mixing 2 μL template DNA (5–10 ng), 25 μL Taq PCR Master Mix (Invitrogen®), 2 μL (10 μM) of each primer, 2 μL bovine serum albumins (0.8 μg $\mu L^{-1}$ final concentrations) and 17 μL of distilled water. PCR amplifications was carried out using a Thermal Cycler (Techne TC-412, Barloworld Scientific, UK) at 95 °C for 5 min initial denaturation followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The final elongation was held at 72 °C for 7 min prior to cooling at 4 °C.

The PCR products were purified using ExoSAP-IT cleanup kit (USB Corporation) according to the manufacturer's instruction. Cleaned PCR products were sequenced by BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturés instruction using reverse primer H1542R.

The partial 16S rRNA gene sequences were aligned using CLUSTALW program in MEGA 6 software [26]. Reference 16S rRNA gene sequences were retrieved from NCBI GenBank database using BLASTn. The phylogenetic relationship of the sequences to closest matches in public database was constructed using Neighbor-Joining Method [27]. The evolutionary distances were computed using the Tamura-Nei method [28] and were in the units of the number of base substitutions per site. The stability and reliability of the relationships of the lineages on the inferred trees was tested by bootstrap analysis [29] for 1000 replicates.

### 2.7. Biodecolorization assay

In order to determine the wavelength of the maximum absorbance, 100 mg/L of RR 239 dye was prepared and scanned in the range of 190 − 800 nm using the UV–vis spectrophotometer. Then, $\lambda_{max}$ of RR 239 dye was considered at one absorbance unit. Calibration curve was also prepared using concentration ranged from 1 − 100 mg/L of RR 239

**Table 2**
Characteristics of the dyes used in this study.

| Color Index Name | Common/Product Name | Molecular Formula | Molecular Weight (g/mol) | $\lambda_{max}$ (nm) |
|---|---|---|---|---|
| Reactive Red 239 | Everzol Red 3BS | $C_{31}H_{19}ClN_7Na_5O_{19}S_6$ | 1136.32 | 541 |
| Reactive Red 141 | Procion Red HE7B | $C_{52}H_{34}Cl_2N_{14}O_{26}S_8$ | 1597.00 | 544 |
| Reactive Red 120 | Evercion Red HE3B | $C_{44}H_{24}Cl_2N_{14}Na_6O_{20}S_6$ | 1469.98 | 535 |
| Reactive Yellow 84 | Procion Yellow HE4R | $C_{56}H_{38}Cl_2N_{14}Na_6O_{20}S_6$ | 1628.22 | 411 |
| Reactive Yellow 160 | Reactive Yellow 160 ME4G | $C_{25}H_{22}ClN_9Na_2O_{12}S_3$ | 818.13 | 415 |
| Reactive Blue 198 | Evercion Blue HEGN | $C_{41}H_{30}Cl_4N_{14}Na_4O_{14}S_4$ | 1304.80 | 520 |
| Reactive Blue 19 | Remazol Brilliant Blue R | $C_{22}H_{16}N_2Na_2O_{11}S_3$ | 626.54 | 594 |

$\lambda_{max}$ = Maximum wavelength.

dye. From the concentration and the measured absorbance data, a calibration curve was constructed. For other six dyes tested, $\lambda_{max}$ and calibration curves were constructed following the same way.

The extent of decolorization was determined by measuring the absorbance (at $\lambda_{max}$ = 541 nm) of the samples at a 24 h interval (i.e. 0, 24, 48, 72 and 96 h). To ensure that all the decolorization were biologically mediated, MSM containing dye without inoculum served as the control was carried out in parallel.

For analysis, 10 mL of the liquid sample was aseptically collected from the reactor every 24 h and centrifuged at 4000 rpm for 40 min. The centrifuged cell-free supernatant samples were measured at 541 nm using the aforementioned spectrophotometer. The percentage decolorization was calculated using Eq. (1):

$$\text{Decolorization}(\%) = \frac{A_o - A_t}{A_o} \times 100 \quad (1)$$

where, $A_o$ = initial absorbance, $A_t$ = absorbance after time t

### 2.8. Effects of different parameters on azo dye decolorization

#### 2.8.1. Effects of different carbon and nitrogen sources on decolorization

Experiments were conducted using different carbon sources such as: glucose, maltose, trisodium citrate and starch (each with 0.5 g/L) and media without carbon source. The concentrations of RR 239 and inoculum were fixed at 100 mg/L and 10% (v/v) inoculum size, respectively.

To evaluate the effects of nitrogen on decolorization activity, organic and inorganic nitrogen sources such as peptone, yeast extract, $NaNO_3$, $NaNO_2$ and $(NH4)_2SO_4$ were added to nitrogen free MSM containing 100 mg/L of RR 239. The concentrations of organic and inorganic nitrogen were 0.01 g/L and 1 g/L, respectively. MSM without yeast and any other nitrogen sources were also prepared and used as a control. The media were inoculated with 10% (v/v) culture and incubated under anoxic condition at ambient temperature.

#### 2.8.2. Effects of different culture conditions on decolorization

The effects of various culture conditions such as agitation, aeration, anoxic and anaerobic states on the decolorization of RR 239 were examined. Agitation was achieved on a rotary shaker running at 120 rpm. Anoxic and aerobic cultures were also achieved by using full volume of the reactor and continuous air supply, respectively. All experiments were conducted at ambient temperature and alkaline pH with an initial dye concentration (RR 239) of 100 mg/L. The residue of RR 239 (UV–vis spectroscopy analysis), cell dry weight [30] and DO (Environmental multi-meter Hatch model 40d, India) were monitored as a function of time.

#### 2.8.3. Effects of pH and temperature on decolorization

To study the effects of pH on decolorization, a range of pH values (6–11) were evaluated. The initial pH values were adjusted using NaOH and HCl. The incubation was conducted in liquid MSM containing 100 mg/L of RR 239.

The decolorization of RR 239 by the best isolate was studied at different temperatures including 15, 20, 25, 30, 35, 40 and 45 °C. Aerobically grown culture (10%, v/v) was used to inoculate RR 239 dye containing (100 mg/L) MSM and incubated in adjustable incubator. The UV–vis spectroscopic measurements were carried out every 24 h.

#### 2.8.4. Effects of dye concentration and dye types on decolorization

To determine the maximum RR 239 concentration that the best isolate could tolerate and its effects on decolorization, experiments with different initial dye concentrations (50, 100, 150, 200 and 250 mg/L) were performed in liquid MSM.

To evaluate the decolorization of the best isolate on dyes other than RR 239, the isolate was exposed to Reactive Red 120, Reactive Red 141, Reactive Yellow 84, Reactive Yellow 160, Reactive Blue 198 and Reactive Blue 19 each with a concentration of 100 mg/L. Each dye types were prepared separately and added to MSM. Then, each preparation were inoculated with aerobically grown culture of the best isolate (10%, v/v). A control group without inoculum was performed for each type of dye preparations. Samples were aseptically collected every 24 h and analyzed.

### 2.9. Statistical analysis

All data were presented as the mean value of three measurements ± standard error. The standard error and significant level were calculated using SPSS version 20.0 software. The paired-sample *t*-test and one-way analysis of variance (ANOVA) with Tukey post hoc test were done to obtain statistical significance between mean values. Pearson correlation analysis was also performed to analyze the relationship between the number of isolates and the physico-chemical values of the lakes. Differences were considered significant if $p < 0.05$.

## 3. Results and discussion

### 3.1. Isolation and characterization of dye decolorizing bacterial isolates

Dye decolorization using alkaliphilic microorganisms was carried out for almost a year (from November 2014 to September 2015). As shown in Fig. 2, a total of 135 morphologically distinct colonies were isolated from Abaya and Chamo Lakes. Since each isolate was not selected based on clear zone formation on dye containing solid media, the decolorization potential of each isolate was examined in dye containing liquid MSM. The isolates which showed color removal within 14 days of incubation were considered as decolorizer (103 isolates) otherwise considered as non-decolorizer (32 isolates). The majority of dye decolorizers (including the best isolate) were obtained from Lake Chamo (78.6%) (Fig. 2). This is most likely related to the physico-chemical characteristics of the lake. Indeed, statistical analysis showed that the two microbial sampling sites were significantly different in pH and salinity. Lake Chamo had higher pH (9.1 ± 0.2) and salinity (1154.5 ± 0.6 mg/L) values than Lake Abaya (Table 1). The numbers of isolates were also strongly correlated with the lakes' pH and salinity.

The color removal efficiencies of 103 decolorizers were varied significantly (5–100%), of which 39 isolates achieved decolorization efficiency ≥91% within 96 h (Supplementary data Table 1). Particularly, seven isolates showed complete decolorization at 24 h, and they were further tested at higher RR 239 dye concentration (50–200 mg/L). After testing, isolate CH12 (hereafter called strain CH12) was found to remove 92–100% and 100% of RR 239 dye at 24 and 72 h respectively,

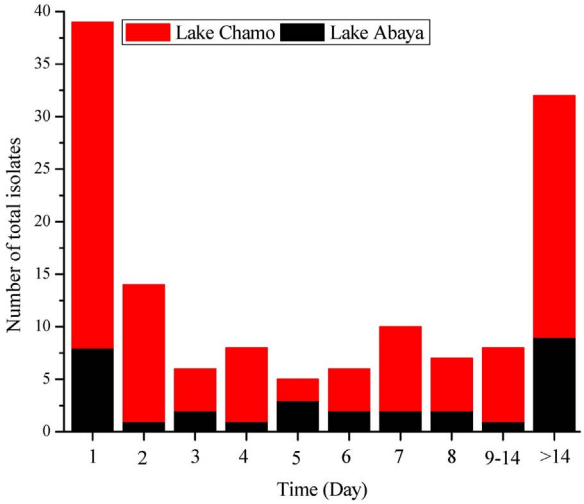


**Fig. 2.** The number of isolates from Lakes Abaya and Chamo over time.

**Table 3**
Morphological, physiological and biochemical characterization of strain CH12.

| Test type | Result |
|---|---|
| Morphology: | |
| Bacterial cell shape | Rod |
| Bacterial colony color | White |
| Bacterial colony form | Irregular |
| Bacterial colony elevation | Flat |
| Gram staining | + |
| Spore staining | + |
| Motility | + |
| Biochemical: | |
| Anaerobic growth | + |
| Aerobic growth | + |
| Catalase | + |
| Oxidase | + |
| Physiological: | |
| Temperature range (°C) | 15–45 |
| Temperature optimum (°C) | 30 |
| pH range | 6–11 |
| pH optimum | 9 |
| NaCl requirement | 0 |
| NaCl optimum (%) | 10 |
| NaCl tolerance (%) | 20 |

which significantly differed from the other six isolates (Supplementary data Table 2).

Morphological and biochemical characterizations showed that strain CH12 was found to be motile, rod in shape, white in color, positive for catalase, oxidase, spore and Gram staining tests. Physiologically, strain CH12 grew in a wide range of temperature (15–45 °C), pH values (6–11) and NaCl concentrations (0–20%), with the optimum being 30 °C, 10 and 10%, respectively (Table 3). As a result, strain CH12 can be categorized under genus *Bacillus* based on these morphological, biochemical and physiological characterizations.

Using 16S rRNA gene sequencing, the taxonomic position of strain CH12 was also determined. The phylogenetic analysis showed that strain CH12 belongs to the domain bacteria particularly to the phylum Firmicutes (Fig. 3). The 16S rRNA gene sequence forms a stable clade with typical strains of all genus *Bacillus*. The strain forms the same branch with *Bacillus* sp. S2, *Bacillus* sp. LCP37, *Bacillus cereus* strain V3, *Bacillus agaradhaerens* strain DSM 8721 and *Bacillus* sp. WL-S20 with a higher 16S rRNA gene sequence similarity (99%). However, in the same phylum with higher gene sequence similarity (99%), the strain forms a distinct tree branch with *Bacillus* sp. ZBAW6. The strain also showed a distinct lineage with *Halomonas venusta* and *Pseudomonas aeruginosa* from another phylum used as an outgroup. Based on 16S rRNA gene sequence similarity, strain CH12 can be grouped in the genus *Bacillus* and designated as *Bacillus* sp. strain CH12. The 16S rRNA gene sequence of *Bacillus* sp. strain CH12 isolated in this study was deposited under GenBank with accession number KU991138.

*Bacillus* strains are ubiquitous in activated sludge and have been found to degrade different dye groups [1,9,16]. Recently, there is also an attempt to use moderately alkaliphilic *Bacillus cereus* for textile dye treatment [31], which is the way to find better fit microbial isolate to the nature of textile effluent.

In order to learn more about alkaline sample inoculum for textile dye treatment, the decolorization efficiency of *Bacillus* sp. strain CH12 was compared with previously reported microbial isolates from uncontaminated non-alkaline, uncontaminated alkaline and contaminated environmental samples (Table 4). In this study, *Bacillus* sp. strain CH12 isolated from uncontaminated alkaline Lake Chamo showed better dye removal efficiency (95–100%) than the *Bacillus* sp. isolated from uncontaminated but non-alkaline (30–47%) environmental samples [19,20]. However, comparable dye decolorization (93–100) was observed from uncontaminated alkaline samples collected in India [32] and China [18] (Table 4). Compared to effluent adapted microbial isolates [3,4,8,33,34], which exhibit a wide range of decolorization efficiency (50–100%), the alkaline lake strain CH12 showed comparable/higher dye removal efficiency (Table 4). This finding clearly

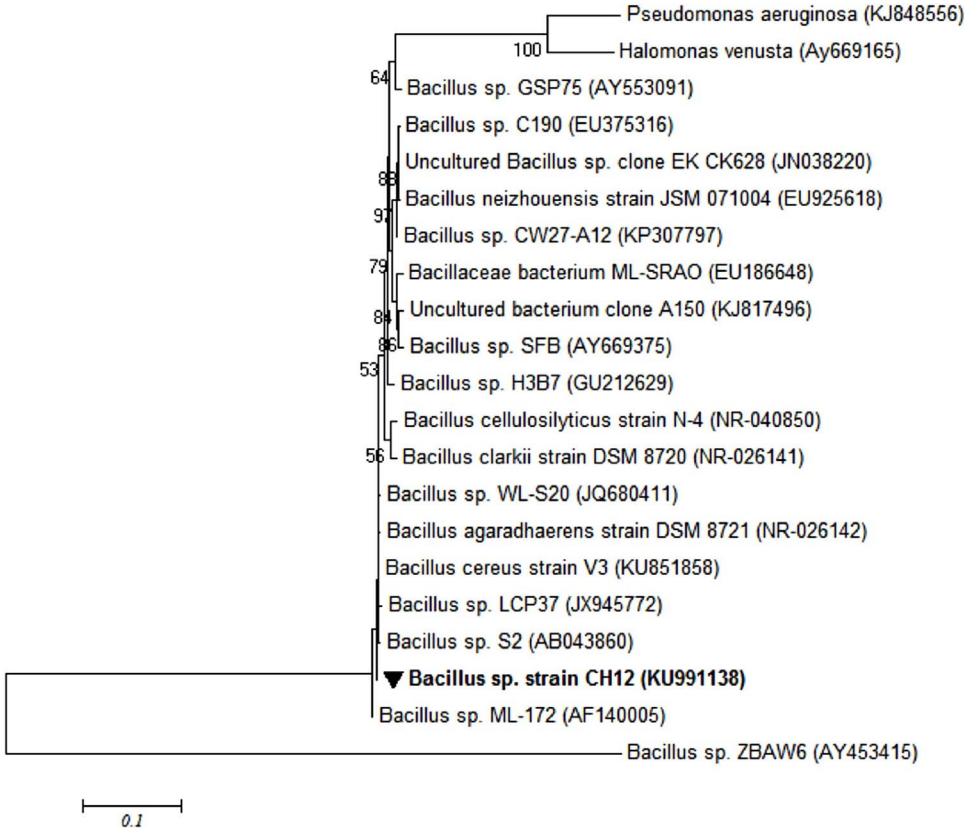


**Fig. 3.** *Bacillus* sp. strain CH12 and related organisms were aligned based on 16S rRNA gene sequences retrieved from NCBI GenBank with neighbour-joining method. The triangle filled indicates strain CH12 isolated from Chamo Lake in this study. Bootstrap values based on 1000 replications are listed as percentages at the branching points (values ≥50% shown at the node). Scale bar, 0.1 is the number of nucleotide changes per sequence position.

**Table 4**
Comparison of textile dye removal efficiency of this study and other studies.

| Microbial isolate/s | Sample type | Dye type (conc., mg/L) | pH | Time (h) | Decolorization (%) | Reference |
|---|---|---|---|---|---|---|
| *Bacillus* sp. strain CH12 | Rift Valley alkaline lake sediment | Reactive Red 239 (100) | 9.0–10.0 | 24–96 | 95–100 | This study |
| *Bacillus* sp. | Non-contaminated soil | Reactive Black B (Nm) | Neutral | 240 | 30 | [19] |
| *Bacillus* sp. (N1 to N6) | Non-contaminated soil | Seven dyes mixed (56) | Nm | 336 | 40–47 | [20] |
| *Bacillus cereus* | Alkaline soda soil sample | Direct Blue 151 (200) | 9.5 | 120 | 93 | [31] |
| *Shewanella haliotis* DW01 | Alkaline Lake water sediment | Reactive Blue 172 (50) | 9.5 | 12 | 93 | [32] |
| *Halomonas* sp. strain GTW | Alkaline coastal sediment sample | Reactive Red K-2BP (100) | 6.5–8.5 | 24 | 98–100 | [18] |
| *Bacillus* sp. strain Ak1 | Dye contaminated soil | Metanil Yellow (200) | 5.5–9.0 | 24 | 99 | [33] |
| *Staphylococus arlettae* strain VN-11 | Textile effluent AS | Four dye mixed (400) | 7.0 | 10–48 | > 97 | [34] |
| *Citrobacter* sp. CK3 | Textile mill AS | Reactive Red 180 (200) | 6.0–10.0 | 24–120 | 70–96 | [8] |
| *Comamonas* sp. UVS | Dye contaminated soil | Direct Red 5 B (50) | 6.0–12.0 | 6–13 | 78–100 | [13] |
| *Paenibacillus larvae* | Textile industry AS | Indigo Carmine (100) | 6.0–8.0 | 4–10 | 88–100 | [12] |
| *Halomonas aquamarina* D2 | Textile industry effluents | Remazol Black B (50) | 5.0–11.0 | 96 | 50–72 | [3] |
| *Pseudomonas aeruginosa* NBAR12 | Dye contaminated soil | Reactive Blue 172 (500) | 7.0 | 42 | 83 | [15] |
| *Aeromonas hydrophila* DEC1 | Textile AS | Red RBN (3000) | 5.5–10.0 | 8 | > 90 | [4] |

AS = Activated sludge, Nm = Not mentioned.

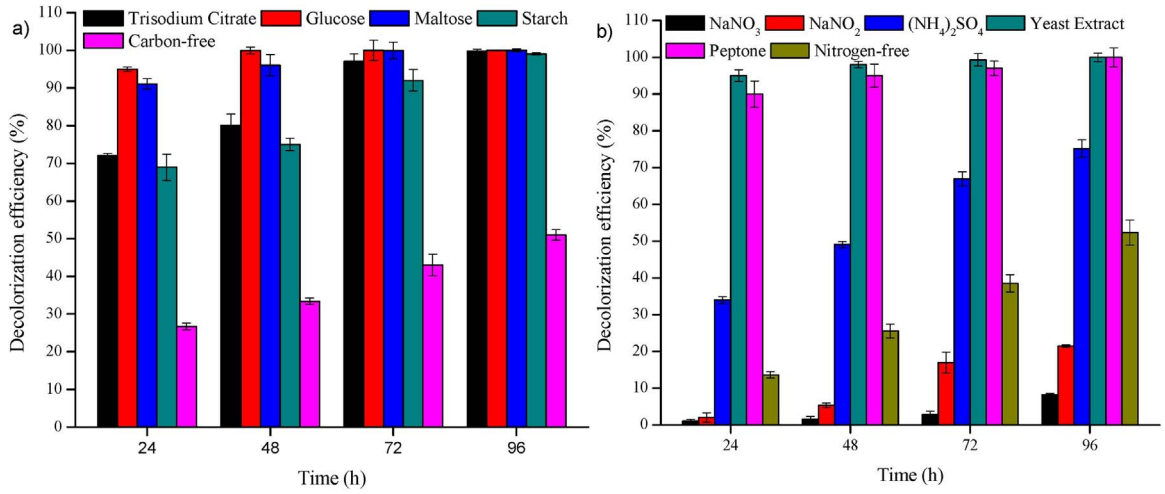


**Fig. 4.** The effects of different nutritional supplements [(a) carbon sources, (b) nitrogen sources] on decolorization efficiency.

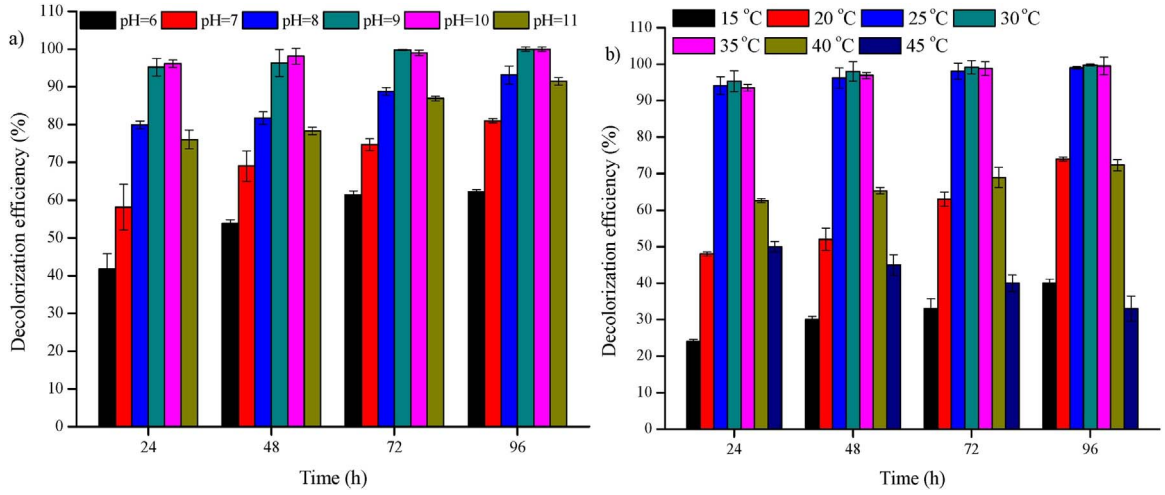


**Fig. 5.** The effects of pH (a) and temperature (b) on decolorization efficiency.

indicated that alkaliphilic microbial isolates could be a better candidate for textile dye decolorization (Table 4). It has been reported that the pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions [21]. Thus, under application condition, using alkaline lake microbial isolate (i.e. *Bacillus sp.* strain CH12) can significantly enhance dye decolorization efficiency by avoiding chemical costs used to re-adjust alkaline textile effluent to neutral pH range, because most traditional textile wastewater treatment systems employ neutrophilic microorganisms that work at neutral pH value.

**Table 5**
Effects of culturing conditions on dissolved oxygen concentration, biomass and dye removal as a function of time.

| Culture condition | Decolorization efficiency (%) | | | | Dissolved oxygen (mg/L) | | | | Dry weight (g/L) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h | 24 h | 48 h | 72 h | 96 h |
| Anaerobic | 90.0 ± 2.1 | 94.0 ± 1.3 | 96.0 ± 0.2 | 100.0 ± 0.1 | 0.20 ± 0.1 | 0.10 ± 0.1 | 0.06 ± 0.0 | 0.01 ± 0.0 | 0.20 ± 0.1 | 2.15 ± 0.3 | 2.64 ± 0.1 | 2.57 ± 0.5 |
| Anoxic | 96.2 ± 1.5 | 99.0 ± 0.8 | 99.7 ± 0.3 | 100.0 ± 0.0 | 0.76 ± 0.3 | 0.46 ± 0.2 | 0.31 ± 0.1 | 0.25 ± 0.0 | 0.88 ± 0.2 | 2.94 ± 0.1 | 3.25 ± 0.4 | 3.29 ± 0.3 |
| Shaker | 2.0 ± 1.0 | 7.5 ± 2.0 | 12.4 ± 1.8 | 18.6 ± 2.4 | 1.33 ± 0.3 | 1.36 ± 0.1 | 1.32 ± 0.2 | 1.35 ± 0.1 | 3.16 ± 0.1 | 3.33 ± 0.4 | 3.41 ± 0.2 | 3.52 ± 0.1 |
| Aerobic | 1.2 ± 0.3 | 2.0 ± 1.5 | 3.5 ± 0.4 | 6.0 ± 0.3 | 2.54 ± 0.3 | 2.35 ± 0.5 | 2.50 ± 0.4 | 2.42 ± 0.2 | 3.49 ± 0.3 | 3.63 ± 0.2 | 3.66 ± 0.3 | 3.58 ± 0.5 |

### 3.2. Effects of different parameters on azo dye decolorization

#### 3.2.1. Effects of different carbon and nitrogen sources on decolorization

Dye decolorization efficiency by strain CH12 was significantly influenced by medium composition. During the entire incubation period of strain CH12, the effect of carbon source on RR 239 decolorization efficiency was found in the order of glucose (95–100%) > maltose (91–100%) > trisodium citrate (72–100%) > starch (69–99%) > carbon-free (27–51%) (Fig. 4a). Compared to carbon-free MSM culture growth, the decolorization efficiencies were significantly enhanced using different carbon sources, which elucidated the requirement of sufficient electron donors for the growth and maintenance of strain CH12. Another possible explanation of the higher decolorization using organic carbon source could be the nutritional contribution of the sources, which resulted in fast growth of the organism. When strain CH12 actively grow, oxygen was depleted and an anoxic environment was created, which might be favorable for the anaerobic reduction of the dye. In the absence of additional carbon sources, dye decolorization efficiency increased from 25% at 24 h to 51% at 96 h, suggesting that the yeast extract might be deaminated and used as a carbon source; or biologically degraded end products of RR 239 might have been used as a carbon source. In addition, the ability of the strain to use starch efficiently presented a practical advantage. In most cases, textile industries use starch for sizing purpose, which will be washed in the subsequent processing steps, leading to excessive concentration of starch in textile effluent [16,35]. In this case, the organisms may not need any input of other additional carbon source to bring about efficient dye decolorization.

The effects of organic and inorganic nitrogen sources are shown in Fig. 4b. Compared to inorganic nitrogen sources, decolorization efficiency was significantly improved for cultures supplemented with organic nitrogen [peptone and yeast extract (≥90%) within 24 h]. During 24 h incubation period, the cultures with $NaNO_3$ (1%, $p = 0.660$) and $NaNO_2$ (2%, $p = 0.127$) showed lower percentage of decolorization than the non-nitrogen supplemented culture (14%), but the difference was not statistically significant. Organic and inorganic nitrogen affected RR 239 decolorization of the strain CH12 in the order of yeast extract (95–100) > peptone (90–100%) > $(NH_4)_2SO_4$ (34–75%) > nitrogen-free (14–52%) > $NaNO_2$ (2–25%) > $NaNO_3$ (1–8%) (Fig. 4b). Visual observation also clearly showed the effects of the different nitrogen source on RR 239 decolorization (Supplementary Fig. S1). The lower decolorization efficiency for the culture supplemented with $NaNO_3$ suggested that nitrate as an electron acceptor might be preferencially consumed by strain CH12. Previous studies have also reported that $NaNO_3$ supplemented culture leads to lower decolorization efficiency [4,11,12].

#### 3.2.2. Effects of pH and temperature on dye decolorization

As shown in Fig. 5a, the decolorization activity of strain CH12 was evaluated by adjusting the initial pH of the MSM from 6 to 11. During the entire incubation periods, optimum decolorization results ($p < 0.05$) were obtained at pH 9 and 10 (≥95.2 ± 2.3%, $p = 0.924$), compared with minimum values at pH 6 (41.8 ± 4.0–62.2 ± 0.6%, $p < 0.05$). At pH 8 (80–93%) and pH 11 (76–91%), strain CH12 exhibited almost similar decolorization efficiency ($p = 0.993$). Strain CH12 performing decolorization best at alkaline pH range has practical importance to develop industrial wastewater treatment/bioprocess that have alkaline nature. Since textile industries use different salt and sodium hydroxide before dyeing steps, the effluents are characterized by high salinity and alkaline medium [21]. The results of this study are consistent with previous findings [4,8]. Chen et al. [4] reported that the most suitable pH for color removal was between 5.5 and 10.0 under anoxic conditions.

Temperature is also one of the most important operating parameter that can influence the growth and metabolic activity of the microorganisms involved in wastewater treatment. It was reported that lower and higher temperature values significantly inhibited the growth of organism and the activity of the enzymes that were responsible for decolorization [4]. In this study, the effects of temperature was

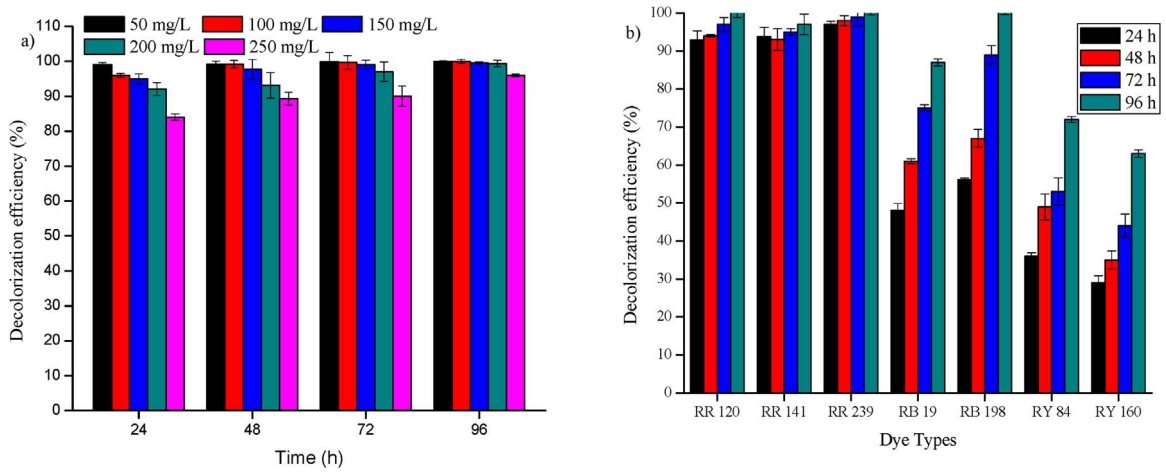


**Fig. 6.** Effects of (a) initial dye concentration and (b) dye types on decolorization efficiency. Reactive Red 120 (RR 120), Reactive Red 141 (RR 141), Reactive Red 239 (RR 239), Reactive Blue 19 (RB 19), Reactive Blue 198 (RB 198), Reactive Yellow 84 (RY 84) and Reactive Yellow 160 (RY 160).

investigated by considering a wide range of temperature values (15–45 °C) and the decolorization results differed significantly. Strain CH12 showed enhanced decolorization when the temperature was increased from 15 to 25 °C, reached the plateau between 25 and 35 °C, and the decolorizing activity was suppressed (50 to 33%) when the temperature further increased to 45 °C (Fig. 5b). This might be due to the loss of cell viability or the deactivation of the enzymes responsible for decolorization [4,8]. The optimum decolorization efficiency of the strain was found at 25–35 °C (94–100%, $p > 0.05$) which favored the growth of mesophilic bacteria. Mesophilic organisms are traditionally used as color wastewater treatment, because treatment at high temperature is considered uneconomical.

### 3.2.3. Effects of different culture conditions on decolorization

Table 5 shows the decolorization efficiency, DO and dry weight results of anaerobic, anoxic, shaker and aerobic conditions. During the entire experimental period, the DO concentrations were found the highest for aerobic culture (2.35 ± 0.3–2.54 ± 0.5 mg/L) followed by shaker (1.32 ± 0.2–1.36 ± 0.1 mg/L). Nevertheless, the strain CH12 incubated under shaker and aerobic conditions showed significantly ($p < 0.05$) reduced decolorization efficiency (2–18.6% and 1.2–6.0%, respectively) compared to the anoxic and anaerobic cultures.

On the other hand, although lower DO values were recorded for anoxic (0.25 ± 0.0–0.76 ± 0.3 mg/L) and anaerobic (0.01 ± 0.0–0.20 ± 0.1) conditions (Table 5), anaerobic and anoxic cultures of strain CH12 contributed to the highest color removal efficiency (90–100% and 96–100%, respectively) within four-day incubation. Compared to the anaerobic cell culture, the color removal by the anoxic culture in the first and second day was better, which might be related to the higher biomass recorded (Table 5). With relatively better oxygen availability, the anoxic culture could use oxygen for rapid proliferation and utilize the dye when oxygen is depleted in the system. The results are consistent with previous findings. For instance, Chen et al. [4] mentioned that *Aeromonas hydrophila* under anaerobic and anoxic conditions showed enhanced Red RBN decolorization efficiency. Compared to agitated culture, *Pseudomonas aeruginosa* incubated without agitation exhibited almost two-fold higher decolorization activity [15]. Other studies also suggested that microbial degradation of azo dyes was often an enzymatic reaction linked to anaerobiosis,and was inhibited by oxygen,which could compete with the azo group as the electron receptor in the oxidation of reduced electron carriers, i.e. NADH [11].

### 3.2.4. Effects of dye concentration and type of dye on decolorization

The dye concentration of textile industry wastewater is commonly in the range of 16–20 mg/L [5]. However, the effect of much higher initial dye concentration (50–250 mg/L) on strain CH12 decolorization potential was evaluated in this study. In the first day of culture incubation, the decolorization efficiency of the strain was found to be ≥96% for lower initial dye concentrations (50–100 mg/L) and 84–95% for higher initial dye concentrations (150–250 mg/L) which was significantly differ ($p = 0.031$) among dye concentrations (Fig. 6a). However, after 96 h of incubation period, strain CH12 exhibited almost equal percentage of decolorization ($p > 0.05$) for all dye concentrations (Fig. 6a). This means that an acceptable high color removal could be achieved by strain CH12 for a wide range of initial dye concentrations. Previous studies showed that dye concentration could influence the efficiency of microbial dye decolorization through a combination of factors including toxicity imposed by higher dye concentrations [15]. However, the results from the present study demonstrated that higher dye concentration (250 mg/L) was not toxic to strain CH12 (Fig. 6a). Thus, this culture may hold great potential for treating industrial wastewater containing high dye concentration.

Textile industries are known to use different types of dyes and the effluents contain different dyestuffs [1,36]. To examine if strain CH12 can degrade other commonly used textile dyes, the culture medium was supplemented with 100 mg/L of six different dyes (Table 2). During the first day of incubation, strain CH12 showed significantly different decolorization efficiency variations for Red (> 90%), Blue (< 60%) and Yellow (< 40%) reactive dyes (Fig. 6b). At the end of the fourth day, decolorization efficiency of the strain was improved (63–100%) for all dye types, suggesting that under application condition strain CH12 could be used to decolorize complex dye effluent with minor acclimation. Similar results (20–100%) were also obtained by Chen et al. [4] with an extended period (seventh day incubation) using *Aeromonas hydrophila* DEC1. The variations of decolorization for different dyes by strain CH12 might be attributable to the structural diversity of the dyes (Fig. 6b). In fact, it has been reported that decolorization variation depends on the structure and complexity of dyes, particularly on the nature and position of substituent in the aromatic rings [1]. For instance, the half-life of hydrolyzed Reactive Blue 19 is about 46 years at pH 7 and temperature of 25 °C [36]. However, strain CH12 showed relatively better decolorization efficiency for Blue dyes (48–100%) than Yellow dyes (30–72%) (Fig. 6b).

## 4. Conclusion

Batch experiments of azo dye decolorization using alkaliphilic microorganisms were conducted under anoxic condition. During the screening activity, strain CH12 was found to be the most efficient decolorizer (92–100%) within the first day of incubation using RR 239 dye concentrations of 50–200 mg/L. The decolorization efficiency of strain CH12 was significantly enhanced when the MSM was supplemented with carbon and organic nitrogen sources. The presence of

nitrate and nitrite significantly reduced the strain decolorization efficiency, indicating that RR 239 dye was not a preferential electron acceptor. Decolorization efficiency of strain CH12 was also found to be the highest when incubated under anaerobic and anoxic conditions than under aerobic condition, suggesting that the process of dye decolorization might involve oxygen sensitive metabolic activities. Strain CH12 exhibited ability of decolorizing seven different types of dyes with elevated dye concentrations which proves the biotechnological potential of this strain for textile effluents treatment.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2017.06.007.

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