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CLARIFICATION OF SALINE TEXTILE DYE WATERS USING HALOARCHAEAL AEROBIC SEQUENTIAL BIOREACTOR SYSTEM

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ABSTRACT

The presence of high concentration of salts in textile industrial effluent waters is a major hindrance to use of conventional activated sludge treatment. Hence, microbial processes functional at high concentration of salts are sought for. We devised an aerobic synthetic bioreactor using Halorubrum saccharovorum strain GUMFAS1 producing cellulase-free xylanase and Halococcus saccharolyticus strain GUFF70 producing xylanase-free cellulase, which requires and resist high concentrations of salt. Individual, whole cells of Halorubrum saccharovorum and Halococcus saccharolyticus gave a decolourization efficiency of 38%, 45%, 58% and 28%, 30%, 45% for methylene blue, malachite green and congo red, respectively. Decolourization could be increased to 40%, 50% and 60% for methylene blue, malachite green and congo red, respectively by using whole cells of both cultures in 1:1 (v/v). Spent media containing 16.4 U/ml of crude cellulase and 11.2 U/ml of crude xylanase could also decolourize the synthetic textile waters in addition to degrading cellulose and hemicelluloses. The mixture of whole cells and crude enzyme supernatants achieved 79% decolourization of mixture of synthetic textile dye waters in four days. Conclusively, the process devised by us is the first report on using archaeal microbes for decolourization of toxic dyes as well as degradation of cellulose and hemicellulose from saline synthetic textile waters.

KEY WORDS

Cellulase, Dye, Haloarchaea, Xylanase

INTRODUCTION

Textile industries are the main contributors of economy in Asia and yet their effluents are major pollutants of water bodies, fauna and flora [1]. They are the largest consumers of synthetic azo dyes with a great variety of colours [2,3]. Disposal of azo dyes into the environment especially from the textile industry is a major threat to human health and environment [4]. Exposure to azo dyes including Congo Red, Methylene Blue and Malachite green causes carcinogenic toxicity and genotoxicity resulting even in birth defects. A high incidence of skin irritation, nausea and vomiting and bladder cancer is reported among dye workers exposed to azo dyes [5]. In addition to dyes, the textile industrial effluents contain 15–20% concentration of salt [6,7] and fines of cellulose and hemicelluloses [8]. Available physical and chemical methods are unable to completely remove azo dyes, are not economical and also produce large quantities of toxic sludge [9]. The conventional biological methods using activated sludge are not compatible, as microorganisms therein cannot withstand high salt of textile effluents [10].

Studies have shown that halotolerant and halophilic bacteria are useful in decolourizing dyes in presence of chloride, carbonate and sulphate which are the common salts used during the reactive dyeing processes

in a textile industry [11,12,13]. As yet there are no reports using Haloarchaeal cultures in clarifying effluent waters containing textile dyes.

In view of this, we used cellulase and xylanase producing *Halorubrum saccharovorum* strain GUMFAS1 and *Halococcus saccharolyticus* strain GUFF70 in a simple aerobic sequential bioreactor system to clarify synthetic textile waters. Individually and in combination the cultures clarified the synthetic textile waters by sorbing the dyes and also degraded the cellulose cotton textile fibres, therein.

MATERIALS AND METHODS

Haloarchaeal Cultures

Two Haloarchaeal cultures, one previously identified as *Halorubrum saccharovorum* strain GUMFAS1 (Accession Number: MG601803) and second isolate GUFF70 isolated from Ribandar salt pans, Goa was used in this study. The medium used for isolation was 25% NaCl Tryptone Yeast Extract (NTYE) Medium [14] (g/L) (Crude salt, 250; $MgSO_4.7H_20$, 20; $CaCl_2.2H_2O$, 0.2; KCl, 5; Tryptone, 5; Yeast Extract, 3), pH 7 and 300 U/ml penicillin. Incubated at 28°C for 20 days. The isolate was maintained on slopes of same medium solidified with 1% agar.

Phylogenetic characterization of GUFF70

Extraction of genomic DNA and its PCR amplification of 16S rDNA sequencing

A single colony of the purified culture was inoculated in 25% NTYE broth medium and allowed to grow. The Genomic DNA was extracted by lysing the cells of a 96-hour old culture broth in sterile distilled water. This Genomic DNA was used as a template for the 16S rDNA gene amplification using the archaeal specific primers A109 (F) 5'ACGGCTCAGTAACACGT3' and 1510 (R) 5'GGTTACCTTGTTACGACTT3' [15]. The PCR reaction consisted of 2mM $MgCl_2$, 2 U Taq Polymerase, 10X Taq Buffer, 10mM of dNTP's (Sigma), 10mM of each primer and 1µl of the template DNA. The final volume of the reaction was made to 50µl using ultra-pure distilled water. The PCR amplification was performed according to the following conditions; Initial denaturation at 94°C for 5mins, followed by denaturation at 94°C for 30 seconds, annealing at 53.5°C for 40 seconds, elongation at 68°C for 60 seconds (35 cycles) and final elongation at 68°C for 5mins [16] (BIORAD T100™ Thermal Cycler). After amplification, the PCR product was resolved on a 1% agarose gel prepared in 1X TAE buffer which was stained with ethidium bromide (10 mg/ml) followed by visualization on Gel documentation system (Syngene G-BOX, BIORAD). The amplified products were further purified using the Q1Aquick PCR purification kit and sequenced using the automated DNA Sequencer (Applied Biosystems). Sequence similarity was performed using BLAST [17] which was accessed via the National Center for Biotechnology Information (NCBI) website. Multiple sequence alignment was performed with MEGA 7.0 [18] by using CLUSTAL W [19] and a phylogenetic tree was constructed using the neighbour joining method [20].

Synthetic textile dye waters (STDW)

STDW's were prepared by adding 250 mg/L of methylene blue, malachite green and congo red individually to 20% NaCl (w/v) solution containing 0.1% (w/v) cotton textile fibres.

Formulation and working of the Sequential Bioreactor (SBR) System

A simple bioreactor was devised as shown in Fig.1. Whole cells were added to 500 mL of STDW, stirred using magnetic stirrer for 96 hours and then allowed to stand (static) at room temperature for 24 hours. The clarified waters were separated from the sludge. This SBR was studied for its performance using whole cells or enzymes or mixture of both.

Clarification of STDW

Haloarchaeal whole cell adsorbent

Seven days old, whole cells of each culture, pregrown in 20% NaCl Synthetic Medium (NSM) (g/L) $MgSO_4.7H_20$, 20; NaCl, 200; $MgCl_2.6H_2O$, 15; $CaCl_2.6H_2O$, 1; KCl, 4; $NaHCO_3$, 0.2; NH_4Cl, 2; $FeCl_3.6H_2O$, 0.005; KH_2PO_4, 0.5; pH 7 [21] supplemented with 0.5% Beechwood Xylan or 0.5% CMC-Na and as mixture of whole cells of the two haloarchaea (1:1) were added to different sets of STDW of methylene blue, malachite green and congo red respectively.

Haloarchaeal Crude Enzymes

Spent medium (culture supernatant) of GUFF70 testing positive for 16.4 U/ml of crude cellulase and GUMFAS1 having 11.2 U/ml of crude xylanase, respectively were separately added to the individual STDW's. Extracellular cellulase and xylanase activity was determined using CMC-Na and Beechwood Xylan, respectively [22]. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1µmol of sugar per minute under the assay conditions.

To determine the decolourization of STDW by cells as well as crude enzymes, the percent decolourization was determined by taking absorbance of the treated and untreated STDW's at λmax of the dye (664nm for Methylene Blue, 616 nm for Malachite Green, 498nm for Congo Red and 600nm for mixed dyes) using the UV-Vis spectrophotometer (Shimadzu) and percentage decolourization was calculated as follows,

% Decolourization = (Initial absorbance - Sample absorbance) x 100
Initial absorbance

To determine that the decolourization was only by the microbial activity and not due to the variation in parameters of light oxidation, abiotic controls were maintained.

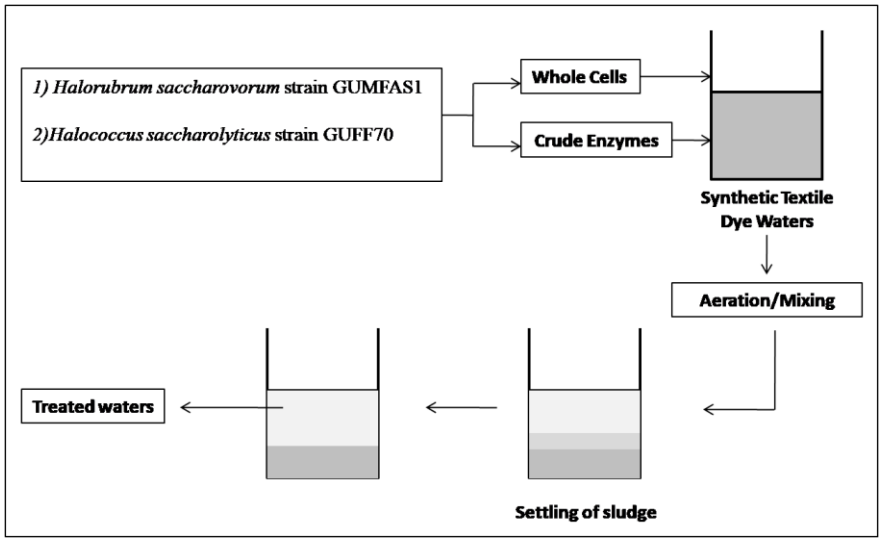

Fig.1: Clarification of synthetic textile dye waters using the devised sequential bioreactor.

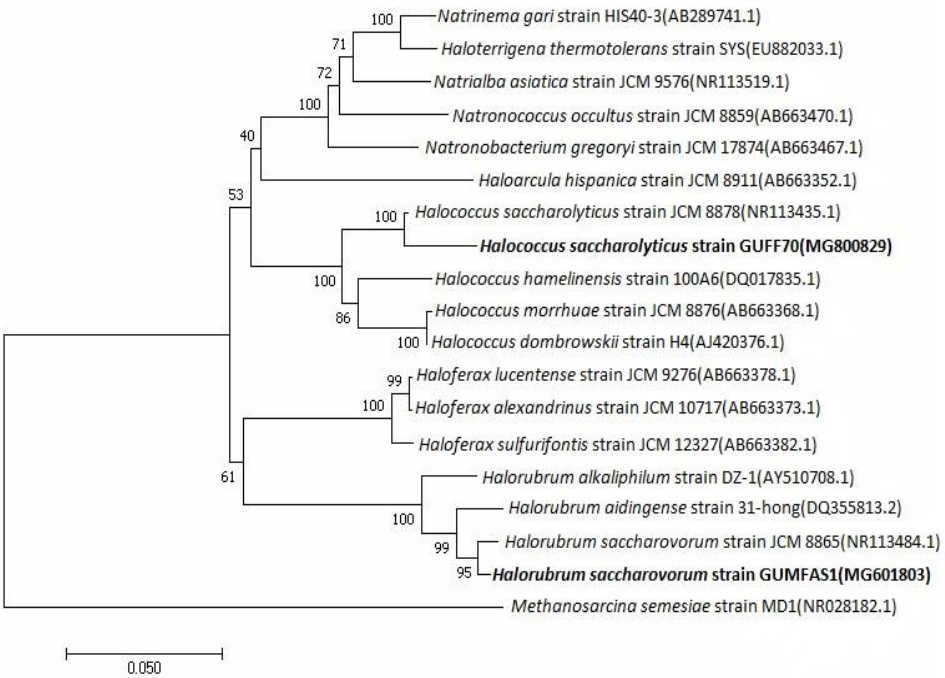

Fig.2: Neighbour-joining tree based on 16S rRNA gene sequences showing evolutionary relationship of GUFF70 with previously characterized species. *Methanosarcina semesiae* MD1 was used as the 'outgroup'. GenBank accession numbers of 16S rRNA sequences are given in brackets. Bar length represent 5 base substitutions per 100 nucleotides. Bootstrap values based on 1000 replicates are shown as percentages at branch nodes.

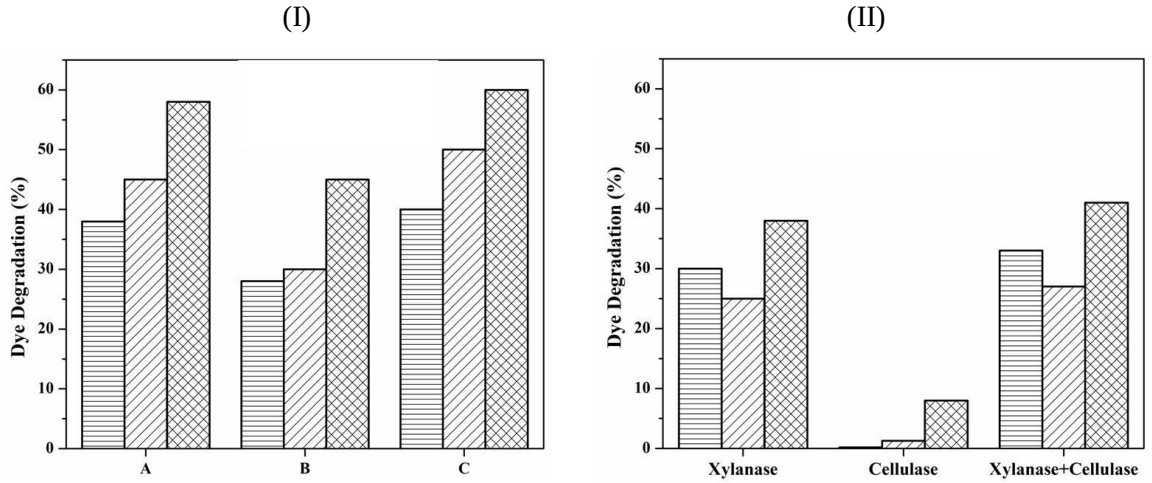

Fig.3: Removal of methylene blue, malachite green and congo red by: (I)whole cells, (A:*Halorubrum saccharovorum* strain GUMFAS1, B:*Halococcus saccharolyticus* strain GUFF70, C: Mixture of A and B); (II) crude xylanase and/or cellulase; : Methylene Blue, :Malachite Green; : Congo Red.

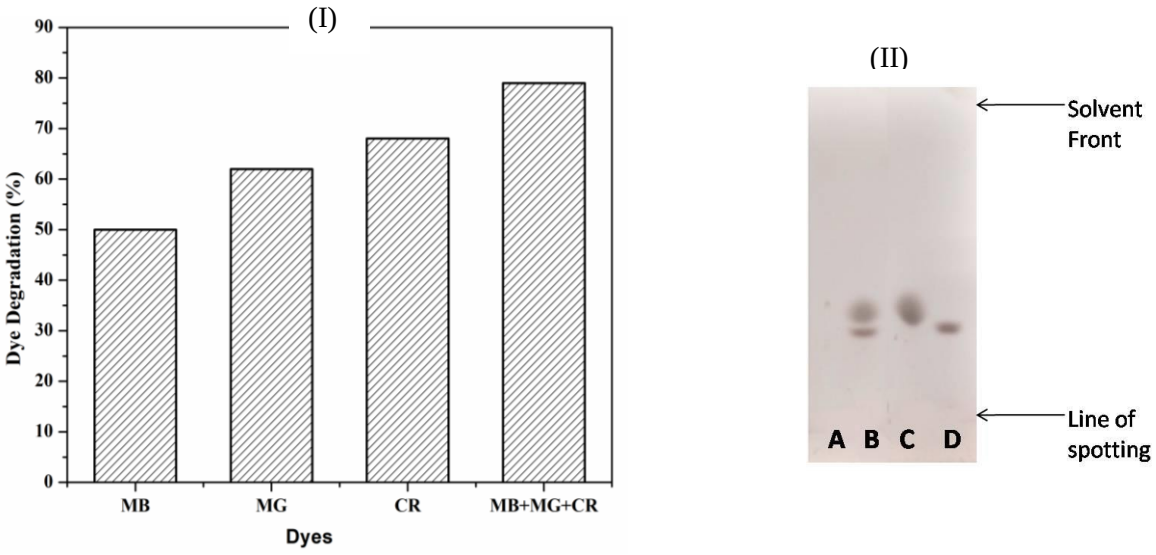

Fig.4: (I)Potential of Dye decolourization by mixture of whole cells and crude enzymes on individual dyes and mixture of the dyes (MB:Methylene Blue, MG:Malachite Green, CR:Congo Red); (II)TLC showing degradation products of cotton textile fibres: A: Untreated STDW; B: Treated STDW; C: Glucose Standard and D: Cellobiose Standard.

Detection of degradation products of cotton textile fibre

Degradation products of cotton textile fibres in presence of dyes were checked by Thin Layer Chromatography (TLC) developed in butanol: acetic acid: water (60:20:20 v/v), followed by spraying with phenol sulphuric acid and heating at 100^{o}C for 10mins till dark purple spots appeared [23]. Rf of each spot was recorded and compared with standard sugars.

RESULTS AND DISCUSSION

Identification of the isolate

Isolate GUFF70 grew as red pigmented colonies. Cells were Gram negative, cocci. Key phenotypic and biochemical characteristics of GUFF70 are summarized in Table1.

Table1: Biochemical characterization of GUFF70 for its tentative identification.

	GUFF70
NaCl range(opt.)(%)	15-30(25)
pH range (opt.)	6-9(7)
Temp. Range (opt.)(°C)	28-50(42)
Colour	Red
Morphology	Cocci
Gram Character	Gram -ve
Carotenoids	+
Motility	+
Catalase	+
Oxidase	+
Agarase	-
Diether lipid	+
Starch hydrolysis	-
Tween 80 hydrolysis	-
Utilization as C and energy source	
D-Fructose	+
D-Galactose	+
D-Glucose	+
Lactose	+
D-Xylose	+
Citrate	-
Reduction of nitrate to nitrite	+
Indole	-
Penicillin	R

(+): Presence; (-): Absence; (R): Resistant

Based on biochemical and phylogenetic characterization, isolate GUFF70 was referred to family Halobacteriaceae, of domain Archaea and as it showed maximum similarity of 98% to *Halococcus saccharolyticus* strain JCM 8878, it was identified as *Halococcus saccharolyticus* strain GUFF70 with an accession number of MG800829 (Fig.2). The culture/isolate GUMFAS1 was previously isolated and identified as *Halorubrum saccharovorum* strain GUMFAS1 with accession number MG601803.

The spent medium of *Halorubrum saccharovorum* strain GUMFAS1 grown with CMC-Na and *Halococcus saccharolyticus* strain GUFF70 grown with Beechwood xylan contained cellulase and xylanase, respectively. Cellulase and Xylanase activity was 16.4 U/ml and 11.2 U/ml, respectively. The whole cells or crude enzymes or mixture of both effectively clarified the synthetic textile dye waters through the sequential bioreactor system.

Efficiency of decolourization of STDW by Whole Cells

Interestingly as seen in Fig.3(I), both cultures showed maximum dye adsorption in the order of congo red > malachite green > methylene blue. Whole cells when used in combination of 1:1 gave a better yield of dye adsorption of 58%, 45% and 60% of methylene blue, malachite green and congo red, respectively as compared to individual whole cells. Thus, indicating that, a mixture of both cultures enhanced the percentage clarification of textile dye waters.

Efficiency of decolourization of STDW by Crude Enzymes

In the second approach, we used the cell-free spent medium containing the crude enzymes. Although cellulase activity was higher as compared to xylanase activity, the percentage clarification of dye waters was lower than with xylanase. In fact, it was just 8% for congo red and negligible for methylene blue and malachite green. Xylanase on the other hand, gave a maximum clarification of 30% for congo red and for methylene blue as seen in Fig.3(II). However, a combination of crude cellulase and xylanase showed higher clarification of waters containing any one of the three dyes. Thus, suggesting that, the mixture of enzymes is more effective in achieving a higher percentage of clarification.

Efficiency of decolourization of STDW by Combination of Cells and Crude Enzymes

Addition of a combination of whole cells and crude enzymes gave a 12%, 17% and 10% increase in clarification of dye waters containing methylene blue, malachite green and congo red, respectively as seen in Fig.4(I). Combination of cells and enzymes also clarified 79% of dye from waters containing methylene blue, malachite green and congo red in the ratio of 1:1:1, respectively. This is a significant result as the textile industries elute a mixture of these dyes in its effluent waters. Additionally, clarification of waters was accompanied by the degradation of cellulose fibres to cellobiose and glucose as detected by Thin Layer Chromatography as seen in Fig.4(II).

Our study using Haloarchaea showed 79% clarification of synthetic textile dye waters, within 4 days. Available reports deal with: i) the use of halophilic and halotolerant bacteria such as *Halomonas* sp. [11,24] and *Halobacillus* sp. C-22 [25] describing azo dye decolorization. ii) the use of *Halogeometricum* sp. strain A and *Haloferax* sp. strain B [26] iii) use of bacterial cell as adsorbent of azo dyes [27,28,29]. . This is the first report on use of biomass and enzymes of *Halorubrum saccharovorum* strain GUMFAS1 and *Halococcus saccharolyticus* strain GUFF70 for effective clarification of synthetic textile dye waters and degradation of cellulose fibres, therein.

CONCLUSION

The ability of whole cells of *Halorubrum saccharovorum* strain GUMFAS1 and *Halococcus saccharolyticus* strain GUFF70 conclusively clarify saline synthetic textile dye waters through the sequential bioreactor system. The azo dye decolourization potential of two haloarchaea was explored in this study. Valuable characteristics of the ability of whole cells of both as well as cellulase and xylanase enzymes produced by them; to decolourize saline synthetic textile dye waters make them good candidates for dye-removal processes from textile effluents. The haloarchaeal ability to decolorize dyes in presence of higher salt concentrations than bacteria is an added advantage for using haloarchaea in remediating dye containing industrial effluents. Therefore, efforts need to be made to develop technology to clarify textile dye industrial effluents.

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