Effective Utilization and Conversion of Spent Distillery Liquid to Valuable Products Using an Intensified Technology of Two-stage Biological Sequestration

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The potential of *Cladosporium cladosporioides* and *Phormidium valdernium* in treating spent distillery liquid in a two-stage sequential step was investigated. During the batch experiment, a maximum decolourisation of 68.5 % and 81.37 % COD reduction was achieved in the first-stage bioreactor. Further, the spent wash from bioreactor was treated with cyanobacteria in the second stage and resulted in COD reduction (3,652 mg L^{-1}) of 89.5 % and 92.7 % decolorization, respectively. Biodegradation was confirmed using HPLC analysis, and the products released during the degradation in the two stages were identified using GC-MS analysis, and found to be 2-octenyl acetate, 1,6-heptadiene from the fungi and oxotetrahydrofuran, hexadecane from cyanobacteria which in turn reveals the fact that the sequential treatment was through the mechanism of biodegradation and not by adsorption. The results imply that sequential treatment using the combination of fungi and cyanobacteria resulted in better degradation and decolourisation for the distillery spent wash.

Key words:
biodegradation, COD reduction, sequential treatment, decolourisation, distillery spent wash

Introduction

In the last decade, studies in pollution control of industrial effluents have become one of the prime concerns of society. Technology problems include enormous quantities of effluents released from industries with a heavy organic load factor and the control strategy implemented. In accordance with the environmental conservation act and rules, it is mandatory to treat wastewater before it is discharged into the environment. The major problem prevailing in the ethanol production industry is the release of large quantities of dark brown distillery effluent, known as spent wash, with high pollutant characteristics remaining after the separation of the product from the fermentation broth. It was reported that spent wash discharge is 15 times the total amount of ethanol produced.[1] The increase in the government policies implemented on pollution control has forced distillery industries to look for an intensified technology for decreasing the effluent characteristics which in turn should be cost-effective and ecofriendly. Several fungi, such as *Phanerochaete chrysosporium* JAG-40, *Aspergillus* sp., *A. gaisen*, *P. pinophilum* and *Emericella nidulans* have been used to reduce distillery spent wash characteristics as an individual basis.[2,3,4] Apart from fungi, marine cyanobacteria are also been used for wastewater treatment. Cyanobacteria are prokaryotic, oxygen evolving, photoautotrophic, gram negative eubacteria having the capability of oxygenic photosynthesis. It was clearly reported that these organisms are widely used in the treatment of wastewater containing organophosphorus pesticides and phenols[5]. Cyanobacteria have the ability to utilize melanoidin present in the spent wash as sole carbon and nitrogen source, and thereby decolorize the spent wash. Another advantage in using cyanobacteria is that, apart from degradation of the melanoidin, it also oxygenates water bodies, resulting in the reduction of the biochemical oxygen demand, BOD and chemical oxygen demand, COD.[5] Investigation in implementing a hybrid method of treating the effluent has gained its validity rather than an individual treatment. In order to increase the biodegradation ability of the process, a two-stage sequential bioreactor was used. As a first stage, the treatment was carried out with *Pseudomonas putida,* followed by *Aeromonas* sp. as a second stage mode.[6] A report indicates that the use of a hybrid technique of electro-coagulation and adsorption is more successful than the individual one.[7] Recently, microbial strains sequential treatment has gained importance in the treatment of distillery spent wash

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due to its high organic load. Fungi were used in the first two stages, followed by bacterial strain as a third stage.[8] Coupled biological treatment in sequence with photochemical processes was only implemented for the reduction of pulp and paper effluent recalcitrant nature.[9] So far, no work has been carried out utilizing both cyanobacteria and fungi in treating the distillery spent wash. Hence, this investigation was focused on reducing the load factor of the distillery spent wash using fungi (*Cladosporium cladosporioides)* in a bioreactor as the first stage, followed by cyanobacteria (*Phormidium valderianum)* in a Tubular Flow Photobioreactor (TFBR) as the second stage.

Materials and methods

Anaerobically treated industrial distillery spent wash (AIDSW)

The molasses spent wash after biomethanation from anaerobic digester was collected aseptically from the distillery division of Bannari Amman Sugars Limited, Periyapuliyur, Erode District, Tamil Nadu, India. The collected spent wash was centrifuged 4200 x g for 15 minutes before use to discard the suspended solids, and stored at 4 °C. The stored AIDSW was filtered and diluted for further studies using distilled water.

Two-stage sequential treatment with fungi and cyanobacteria

A two-stage sequential treatment of AIDSW was carried out with fungi as the first stage, followed by cyanobacteria as the second stage (Fig. 1). During the biodegradation process, the released CO_2 passed through a gas mix to the tubular flow photobioreactor for the growth of cyanobacteria biomass and its subsequent treatment.

Seed culture conditions for bioreactor and photobioreactor

In a 250-mL Erlenmeyer flask, the fungal inoculum was prepared by allowing the culture to grow in 100 mL of liquid potato dextrose broth. Further, the flask was incubated in an orbitary shaker for a period of ten days at a constant temperature of 35 °C and speed of 250 rpm until spores formed. Reseeding was carried out at an interval of 25 days to maintain the active population. After day 5, 2 mL of the culture (containing approximately $8 \cdot 10^6$ spores in one mL) were inoculated into two 500-mL conical flasks, and incubated in an orbitary shaker at 250 rpm for 10 days, which was the seed culture for the first-stage treatment. Cyanobacteria required for the second-stage treatment in TFBR was obtained from the stock culture maintained in Mud Pot Reactor (MPR).

Treatment of AIDSW in first-stage bioreactor

The experimental setup for the sequential process is shown in the (Fig. 1). The experiment was performed in a controlled glass type *in-situ* bioreactor (Bio-Engineering, Model: KLF-2000-W-21339, Switzerland) made of glass (0.130 m inner diameter, height of 0.50 meter and 7.5 Liter working volume). The bioreactor consisted of a double Ruston disc turbine impeller blade attached with disc of 0.0854 m diameter, 0.125 m width of impeller blade, length of 0.165 m, and a space of 24 m between each turbine impeller). The reactor was also fitted with four rectangular baffles of dimensions (0.01 x 0.42 m). The air sparger was placed at the bottom of the reactor at a distance of 0.2 m. The process parameters optimized using single factorial experimental design[10] were scaled to 7.5 L bioreactor with working volume of 3.5 L. Initially, AIDSW was fed into the large-scale bioreactor from the col-

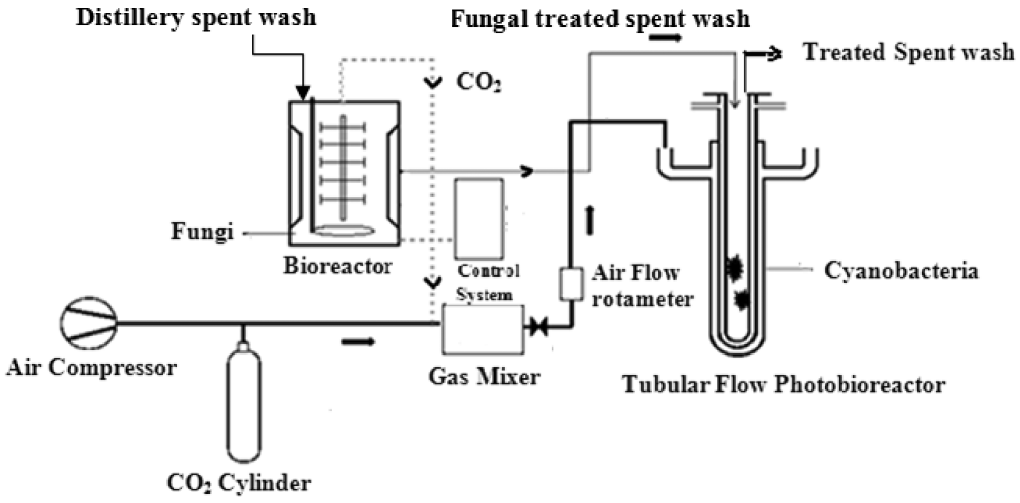

Fig. 1 – *Representation of AIDSW treatment using fungi (first stage), followed by Cyanobacteria (second stage)*

lection tank. The transferred medium contained fructose – 7 g L^{-1}, peptone – 2 g L^{-1}, and pH was adjusted to 6. Nearly 10 % (w/v) fungal inoculum ($8 \cdot 10^6$ spores mL^{-1}) was transferred into the reactor, and the temperature was adjusted to 35 °C, based on the results of RSM studies. The process conditions, like pH, temperature, rpm, air flow rate, dissolved oxygen, and biomass weight inside the bioreactor required for the biodegradation process was monitored and maintained by the microprocessor-based control system attached to the bioreactor. The air flow rate was measured with a precalibrated rotameter and maintained at 1 vvm. Further, the treated AIDSW was again treated using cyanobacteria (*P. valaderium*) in TFBR as the second stage. In the first stage of the bioreactor, the process duration was seven days, followed by ten days in the second stage of the photobioreactor. The CO_2 outlet of the bioreactor was connected to the gas mixture tank, which in turn was connected to the photobioreactor for the growth of cyanobacteria.

Treatment of AIDSW from bioreactor in second-stage photobioreactor

The AIDSW was treated in the bioreactor as the first stage using fungi (*C. cladosporioides*) after centrifugation for 10 minutes at 10,000 x g, and the supernatant was filtered through Whatman No. 1 filter paper and transferred to the photobioreactor. The fully-grown culture (5 g) from the MPR was inoculated into the photobioreactor containing spent wash and artificial seawater nutrient culture medium of half salinity (ASN-III) medium. The biophotosynthetic process parameters, such as pH, temperature, and light intensity were optimized using single factorial experimental design in flask level. Then it was scaled to 2 L with a working volume of 1 L TFPBR. The process conditions in the photobioreactor, such as pH, temperature, and light intensity were maintained at pH 8, 28 °C and 36 W m^{-2}, respectively, which were obtained through optimization studies designed using RSM with central composite design (CCD). During the sequential stepwise AIDSW treatment in the bioreactor followed by photobioreactor, the samples were withdrawn every 4 hours to determine the percentage of decolourisation and COD analysis. The samples were withdrawn from the bioreactor by creating a back-pressure by closing the sterilization outlet valve. The bioreactor had a built-in automatic dissolved oxygen probe, pH probe, and was mounted in a weighing balance, which was used to measure the amount of microbial growth. The CO_2 from the bioreactor during the metabolism of the reaction was analyzed using a CO_2/O_2 gas analyzer (New Brunswick Scientific, EX-2000, USA) attached to the bioreactor after the control system. The initial pH of the medium solution inside the photobioreactor was measured by Digital portable pH meter. The light intensity incident on the column surface was measured by luximeter (Minipa MLM 1010, country). Consequently, the samples collected through peristaltic pump (Miclins, India) were sent to the microbiology and instrumentation laboratories for analysis of the morphological and rheological characteristics of the fermentation broth. Viscosity was measured using Brookfield programmable LVD VII digital viscometer[11].

Biodegradation analysis using HPLC

The samples collected from both bioreactor and photobioreactor were monitored by high pressure liquid chromatography, HPLC, to determine the biodegradation efficiency of the fungus and cyanobacteria. HPLC analysis was carried out at the quality control and assurance laboratory, Centre for Pharmacognosy & Pharmaceutics FRLHT, Bangalore, India. Decolourisation of AIDSW was monitored by HPLC (Shimadzu –LC20AD). 10 mL of samples were taken, centrifuged, and filtered through 0.45 μm membrane filter. The filtered samples were analyzed using mobile phase consisting of acetonitryl and methanol (45:55) (HPLC grade) with 1 mL glacial acid and 0.5 mL sodium acetate.[12,13] The samples were eluted using C-18; reverse phase column of 5 μm SGE, 250 x 4.6 mm SS. The samples were analyzed with UV–detector at 475 nm. The flow rate of the mobile phase was 1 mL min^{-1}.

Laccase enzyme from *C. cladosporioides*

Laccase enzyme was extracted by the Three Phase Partitioning (TPP) method.[14,15] After 7 days of incubation, the medium was withdrawn from the bioreactor, filtered, and the supernatant (crude enzyme) was collected for the extraction of laccase. The process of enzyme extraction was conducted by saturating the crude enzyme solution with ammonium sulphate (w/v) and by adding *t*-butanol (v/v) in the ratio of 1:1 (ratio of crude enzyme extract to solvent). At the end of an hour of incubation at room temperature, the mixtures were centrifuged at constant speed of 10,000 x g for 10 minutes at 25 °C. The precipitate obtained after centrifugation was dissolved with 0.4 mL of 50 mM acetate buffer (pH 4). The aqueous phase and precipitate were subjected to protein estimation and enzyme activity. Further, the influence of ammonium sulfate saturation (20, 30, 40, 50, and 60 %, w/v) to the partitioning behavior of laccase was analyzed.[16] Laccase activity was determined using Syringaldazine. Laccase activity was qualitatively determined by inoculating *C. cladosporioides* in a PDA plate containing 0.02 %

guaiacol. The inoculated plate was incubated in dark conditions at 30°C for about 7 days.[17] The enzyme activity was calculated in U mL^{-1}.

Laccase enzyme from cyanobacteria

The culture from TFPBR was washed with ASN III medium repeatedly and sonicated using an ultrasonic processor (Sonics, USA) to disrupt the cell membrane. Further, the sonicated contents were centrifuged for 10 minutes at 10,000 x g. The laccase enzyme was assayed for its activity as per the protocol followed for *C. cladosporioides*.

GC-MS analysis

During the biodegradation of the distillery spent wash in the sequential stepwise process in the bioreactor and photobioreactor, the samples from each stage were analysed using Gas Chromatography –Mass Spectroscopy GC-MS analysis for the metabolites compounds produced. Before the analysis, the samples were processed following the protocol of.[18] Effluent samples were collected from each stage and subjected to centrifugation for removal of suspended solids and microbial solid broth. Further, the supernatants were adjusted to pH 2 using 0.1 N of HCl. The metabolites were extracted with ethyl acetate and methanol consecutively using soxhalt apparatus. The formed organic layer was collected, dewatered using anhydrous $NaSO_4$, and finally filtered. The residues were dried by passing a stream of nitrogen gas. 100 µL dioxane and 30 µL pyridine were added to the extracted sample followed by silylation with 50 µL trimethylsilyl and trimethyl chlorosilane. The mixture was heated at 60 °C for 15 minutes, and placed in the shaker to dissolve the residues. The analysis was carried out using GC-MS (Thermo GC-TRACE ULTRA VER: 5.0, Thermo MS DSQ-II) equipped with a capillary column (DB -35-Non-polar column 0.25 µm film thickness x 0.25 mm id x 30 m). One micro-liter of each extract was injected to analyse at preset conditions of 40–270 °C. The carrier gas used was helium with a flow rate of 1.0 mL min^{-1}. The data obtained was compared with the National Institute of Standards and Technology (NIST) library inbuilt standard chemical library system of GC-MS.

Results and discussion

The liquid wastes obtained during the molasses fermentation for ethanol production is a highly recalcitrant waste product, and contains a polymer known as melanoidi, which is formed by Maillard amino-carbonyl action. Isolation and screening of fungus were carried out as per the protocol reported by[18]. 18S rRNA partial sequence of strain *C. cladosporioides* was submitted to the National Center for Biotechnology Information, NCBI, and the nucleotide database accession number JN592511 was assigned from the Gene Bank, Bethesda, Maryland, USA.

Treatment of AIDSW in first-stage bioreactor

Biodegradation and decolourisation of distillery waste liquid by fungi and cyanobacteria were tested in a two-stage sequential manner. Fig. 2 represents the COD reduction and residual colour during the course of time in the first stage of the sequential treatment. Colour containing compounds present in the effluent were degraded using lignolytic enzymes.[20] When *E. nidulans* was used for decolourisation, it was observed that peroxidase was responsible and proved to be significant.[21] The results indicate that during the initial period of 2 days, there was no significant change in the percentage of decolourisation. However, on day 7, it was observed that a maximum decolourisation of 68.5 % and 81.37 % COD reduction (Initial COD is 34,800 mg L^{-1} final 6,482 mg L^{-1}) was attained, after which it remained constant. A change in morphology of the organism was noticed from mycelia to pellet adhering to the walls of the impeller and sparger. From the morphological analysis, it was observed that the pellet was broken on day 5, and completely changed into filamentous form from day 7 onward, and this might be one of the reasons for achieving the maximum result. During the initial phase of growth, the organism easily utilizes the available carbon source added as medium, and later on starts to degrade spent wash components for carbon source. Although

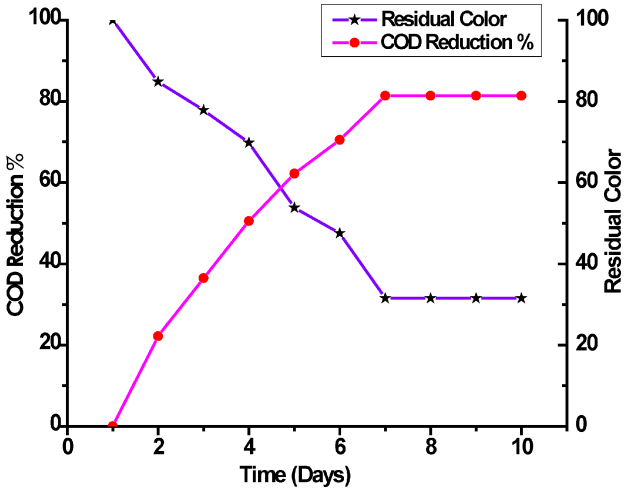

Fig. 2 – *COD reduction and residual color during the course of time in the first stage of the sequential treatment at pH 6 and 35 °C*

spent wash contains large amounts of sugar, the availability of metabolizable simple sugar was almost negligible. Initially, the addition of a readily available external carbon source, like fructose, made it convenient for its metabolism. The presence of laccase and cellulase enzymes in the fungus was also one of the reasons for the degradation and decolourisation of the spent wash.

Treatment of AIDSW from bioreactor in second stage photobioreactor

When the spent wash from the bioreactor was subsequently treated with cyanobacteria as the second stage in a large scale TFPBR, observed was a 92.7 % decolourisation, and 89.5 % COD reduction (3,652 mg L^{-1}) (Fig. 3). The reason for the high ability of sequential treatment was that the load factor of the spent wash was reduced when treated in two stages. This reduction is due to the presence of laccase enzyme present in the cyanobacteria, which was confirmed through enzyme study reported.[15] A similar study was carried out with *E. nidulans (*fungus) and *N. intermediate* (fungus) and *Bacillus* sp., in a sequential three-stage bioreactor, and resulted in 82 % decolourisation with 93.5 % reduction in COD.[8] The application of two bacterial strains of *P. putida* and *Aeromonas* sp. in a two-stage batch culture mode bioreactor resulted in 44.4 % and 60 % reduction in COD and colour, respectively, of the distillery spent wash.[6] However, in another report, it was mentioned that degradation of paper mill effluent was enhanced to 81 % through photocatalytic treatment.[9]

Fig. 4 (a) and (b) shows that the control samples were found to have no changes in the decolourisation and reduction in COD without the inclusion of inoculum. The significant change in color

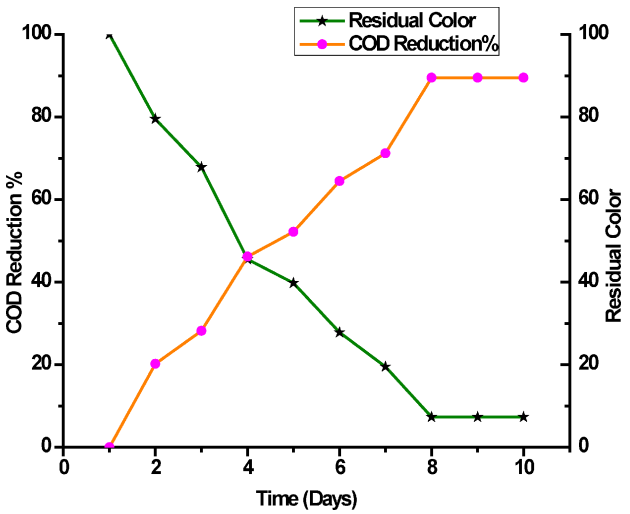

Fig. 3 – *COD reduction and residual color during the course of time in the second stage of the sequential treatment at pH 8, 28 °C and 36 W m^{-2}*

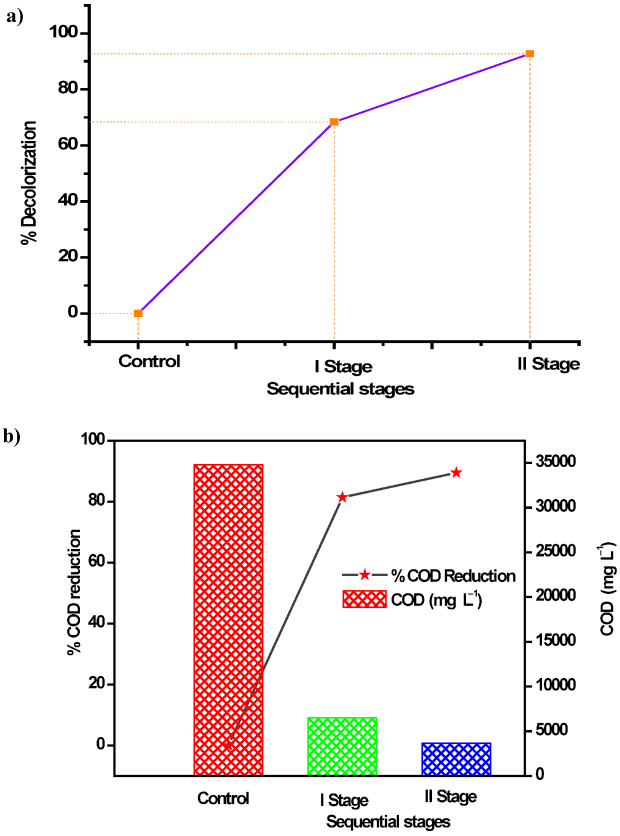

Fig. 4 – *a) Sequential stages and the % decolourisation with control, (b) Sequential stages and COD reduction with control*

and COD reduction of the effluent after fungi and cyanobacteria was due to the bioaccumulation process. The reduction in COD was because the complex higher molecular compounds present in the spent wash were broken and converted into low molecular weight compounds by the presence and segregation of enzymes (laccase and cellulase in the fungi). A similar study was reported earlier, stating the significant role of enzyme in breaking the complex compounds to low molecular weight compounds.[22] Alkaline solution favored adsorption of spent wash on the surface, resulting in high color removal and followed by biological degradation. This is due to the concept of zero point of charge for cyanobacterial species, where the surface seems to be positively charged in acidic conditions and negatively charged in alkaline conditions. A similar study was reported for adsorption and biological decolourisation of Malachite green dye[23,24]. A maximum decolourisation of 68.4 % and 81.37% COD reduction (6,482 mg L^{-1}, initial COD is 34,800 mg L^{-1}) was achieved during the first stage, and a maximum decolourisation of 92.7 % and 89.5 % COD reduction (3,652 mg L^{-1}) during the second stage. Characteristics of the spent wash are shown in Table 1.

Table 1 – *Physicochemical characteristics of the spent wash before and after treatment*

	Before treatment	After treatment (First stage)	After treatment (Second stage)
Colour	Greenish dark brown	Light brown	Very light brown
Odour	Burnt sugar	Less Burnt sugar	Less Burnt sugar
pH	7.20	6	8
Biochemical oxygen demand (BOD) (mg L^{-1})	5000–6500	3800	3200
Chemical oxygen demand (COD) (mg L^{-1})	34,800	6,482	3,652
Total dissolved solids (TDS) (mg L^{-1})	4500–4620	3500	3000
Sulphates (mg L^{-1})	160	140	116
Potassium (mg L^{-1})	850	850	850
free chlorine (mg L^{-1})	800	800	800

Biodegradation analysis using high-pressure liquid chromatography (HPLC)

The HPLC analysis report representing the area, height, retention time, before and after sequential treatment of AIDSW, confirms the biodegradation ability of the fungus on AIDSW (Fig. 5a, b and c). The results indicate that the area and the height of the peak had decreased more in the treated sample than in the untreated sample, which contributes to the fact that sequential treatment was through the mechanism of biodegradation and not by adsorption. A similar confirmation was revealed during the decolourisation and degradation of reactive blue by *Aspergillus* sp.[25] It was observed that the rate of biodegradation was high with fungi, and color reduction is high for cyanobacteria. It was mentioned in the report that the spent wash concentration above 12.5 % (v/v) reduced the COD reduction ability of *Aeromonas* sp.[6] But in this report, it was proved that at 16 % (v/v) concentration of the spent wash treated by fungi was highly utilized by cyanobacteria for its growth with COD reduction ability. This is mainly due to the sequential method adopted in this system. The results implied that stepwise treatment using the combination of fungi and cyanobacteria resulted in better degradation and decolourisation when compared to other single-stage treatment.[10]

GC-MS analysis

The distillery spent wash, after sequential treatment in two stages using fungi and cyanobacteria, was analyzed in detail using GC-MS**.** Complex polymers had produced due to the reaction between amino carboxyl groups that are recalcitrant in nature and exist in molasses and other agri-based industrial waste liquid.[26] The process parameters of 8pH, 36 W m^{-2} light intensity and 30 °C temperature were optimized in conical flask level, before use in the photobioreactor.[27] Microorganisms are known to serve as an important tool for the production of valuable compounds during the degradation of distillery spent wash. The GC-MS chromatogram of the valuable compounds remaining after biodegradation of the distillery spent wash by *C. cladosporioides* is shown in Fig. 6a, and with *P. valdernium* in Fig. 6b. The identified metabolites and degradation products formed during the spent wash treatment in two stages are shown in Table 2 and Table 3. It was observed from NIST database that some valuable compounds, 8-nonen-1-Ol (used to produce 9-bromo-non-1-ene), 2-octenyl acetate (flavor and fragrance agents), 1,6-heptadiene (CAS) (used as a starting reagent in asymmetric synthesis of all stereoisomers of 6-methylpipecolic acids), (cyanomethyl1)cyclohexane (an intermediate in the synthesis of gabapentin), N-ethyl methylketene imine (nucleophilic reagents for organic transformations), a-4,4-bis(2,3-epoxypropyl)pent-1-ene) (a metabolite of bisphenol A (BPA) has potent estrogenic activity *in vitro* and *in vivo*, about 1,000 times more than BPA), 4-methylhex-5-en-1-al (stereoselective synthesis of trisubstitutedtetrahydropyrans), 5-bromo-1-hexene an important raw material and intermediate used in organic synthesis,) and 1-(hydroxymethyl)-3-methylene-1cyclobutanol (addition of iodine fluoride to this compound leads to broad-spectrum antiviral activity, especially against human cytomegalovirus) from the first stage, and 2,7-diamino-8-methyl-5(3,4,5-trimethoxyphenyl)-pyrido[2,3-d]pyrimidin-4(8H)-one, (used as intermediates for the production of pharmaceuticals), bis[N,N-bis(dimethoxyethyl)-N-methylamine]-zirconium, 4-methyl-N-(m-chlorophenyl)-5-vinyl-1,3-oxazolin-2-one, (organometallic precursor compounds use for preparing dielectric thin films), (R)-5-(1-decenyl)-2-oxotetrahydrofuran (pesticide products containing nuranone) and isomeric 4-hexadecenes (polyalphaolefin synthetic lubricant basestock (PAO), and to make surfactants in a blend

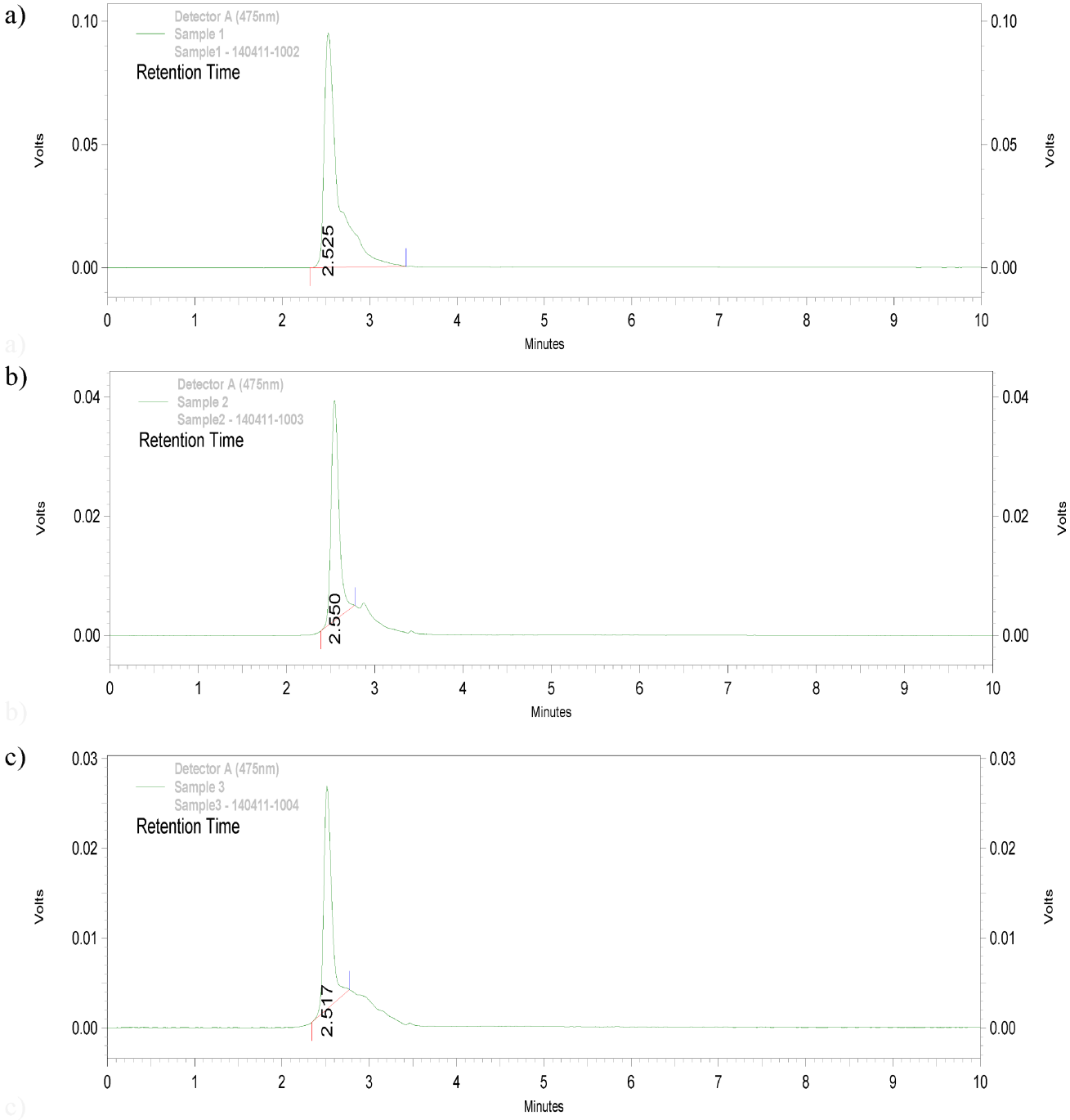

Fig. 5 – *a) HPLC chromatogram for AIDSW before treatment showing a maximum peak with 2.525 retention time, 1072559 area and 95206 height b) HPLC chromatogram for ADSW after treatment with Cladosporium cladosporioides showing a maximum peak with 2.550 retention time, 227827 area and 36858 height c) HPLC chromatogram for ADSW treatment with Phormidium valderianum showing a maximum peak with 2.517 retention time, 155112 area and 24903 height*

with higher linear alpha olefins) from the second stage have been produced during the metabolic reaction, which can be further purified to add market value. The effluent, after the first and second stages of treatment, was analyzed using GC-MS for determining the degradation and metabolic compounds from the spent wash. The result of the study suggested that the disappearance of major peaks after retention time (RT) at 30.475 minutes to RT at 41.946 minutes, and emergence of varied and an increased number of peaks even before RT at 12.084 minutes, which was the first major peak detected in the control (untreated) sample. Some of the degradation products were identified from the available standards of the NIST library. Very few products were degraded in the second stage, as the cyanobacteria had more adsorption property than degradation.

Table 2 – *Identified product released during the degradation of spent wash in the first stage using C. cladosporioides (fungi)*

RSI	Compound name	Molecular formula	Molecular weight
833	4-methylenebicyclo[5.3.0]-2-oxadecane-3-one	$C_{10}H_{14}O_2$	166
832	8-nonen-1-ol	$C_9H_{18}0$	142
906	2-octenyl acetate	$C_{10}H_{18}O_2$	170
841	1,6-heptadiene(CAS)	C_7H_{12}	96
849	(cyanomethyl1)cyclohexane	$C_8H_{13}N$	123
807	N-ethyl methylketene imine	C_5H_9N	83
884	2,7-octadieniol acetate	$C_{10}H_{16}O_2$	168
857	but-4-enyl but-3-ene-1-sulfonate	$C_8H_{14}O_3S$	190
842	but-3-enyl Prop-2-enesulfonate	$C_7H_{12}O_3S$	176
852	6-hydroxy-hexanenitrile	$C_6H_{11}NO$	113
877	a-4,4-bis(2,3-epoxypropyl)pent-1-ene	$C_{11}H_{18}O_2$	182
814	3,5-trans-3(methylsulfonyloxy)-5-[(E)-1-pentenyl]-4,5-dihydro-2(3H)-furanone	$C_{10}H_{16}O_5S$	248
824	4-methylhex-5-en-1-al	$C_7H_{12}O$	112
818	5-bromo-1-hexene	$C_6H_{11}Br$	162
839	1-(hydroxymethyl)-3-methylene-1cyclobutanol	$C_6H_{10}O_2$	114
853	cyclohexy(2-methylenecyclopropyl)carbinol	$C_{11}H_{18}O$	166
839	cis-1-bromo-2,2,3-trimethylcyclopropane	$C_6H_{11}Br$	162
807	2,5-divinyl-tetrahydrothiophen-1,1-dioxide	$C_8H_{12}O_2S$	172
851	a-4,4-bis(2,3-epoxypropyl)pent-1-ene	$C_{11}H_{18}O_2$	166
985	(1R,2R,3S,4Z)-1-(4'-methyl-2'-trichloromethyl-2'-oxazolin-4'-yl)-1-hydroxy-2,3-O-isopropylidene-11-(2"-hexyl-1',3"-dioxolane-2-yl)-4-ene-2,3-undecanediol	$C_{28}H_{46}C_{13}NO_6$	597

Table 3 – *Identified product released during the degradation of spent wash in the second stage using P. valderianum*

RSI	Compound name	Molecular formula	Molecular weight
599	(+-)-10,11-dimethoxycorynan-17-ol	$C_{21}H_{30}N_2O_3$	358
737	1-(2,6-diisopropylphenylimino)-4-(t-butyldimethylsilox)but-2-yne	$C_{22}H_{35}NOSi$	357
733	7-N-ethylanilinopyrrolo[3,4-c]pyridine-1,3-dione	$C_{22}H_{19}N_3O_2$	357
726	4,6-bimethoxy-7-formyl-2,3-dephenylindole	$C_{23}H_{19}NO_3$	357
669	(2R*,8aS*)-1-benzyl-3-isopropylidene-4-methyl-2-phenyl-1,2,3,5,6,7,8,8a-octahydroquinolone	$C_{26}H_{31}N$	357
603	dimethyl (N-methylphenothiazine-2,7-diyl)diacetate	$C_{19}H_{19}NO_4S$	357
600	dihydroconessine	$C_{24}H_{42}N_2$	358
670	2,7-diamino-8-methyl-5(3,4,5-trimethoxyphenyl)-pyrido[2,3-d]pyrimidin-4(8H)-one	$C_{17}H_{19}N_5O_4$	357
610	2,4-dihydro-8-methoxy-4,4-dimethyl-2-phenyl-1H-[1]benzopyrano[4,3,2-cd]indol-1-one	$C_{23}H_{19}NO_3$	357
615	O-methyllimousamine	$C_{20}H_{23}NO_5$	357
608	2-(3,5-di-tert-butyl-4-(trimethylsilox)phenyl)-N-methylpyrrole	$C_{22}H_{35}NOSi$	357
674	5-azido-2,3-O-cyclohexylidene-5-deoxy-D-ribono-1,4-lactone	$C_{11}H_{15}N_3O_4$	253
737	N-phenylimide-3-ferrocenyl-4,5-(piperidine-1,4-diyl)1,2-diaza-.dealta.(4)-hydrophalic acid	$C_{27}H_{26}FeN_{402}$	494
725	bis[N,N-bis(dimethoxyethyl)-N-methylamine]-zirconium complexe	$C_{22}H_{46}N_{204}Zr$	492
725	bis[N-methyl-N-di-(	$C_{22}H_{46}N_{204}Zr$	492
650	4-methyl-N-(m-chlorophenyl)-5-vinyl-1,3-oxazolin-2-one	$C_{12}H_{10}CINO_2$	235
724	2-(1-decenyl)-tetrahydrofuran	$C_{14}H_{26}O$	210
610	(5Z)-4-methylene-N-(p-chlorophenyl)-5-propylidene-2-oxazolidinone	$C_{13}H_{12}CINO_2$	249
655	(R)-5-(1-decenyl)-2-oxotetrahydrofuran	$C_{14}H_{24}O_2$	224
657	-isomeric 4-hexadecenes	$C_{16}H_{32}$	224

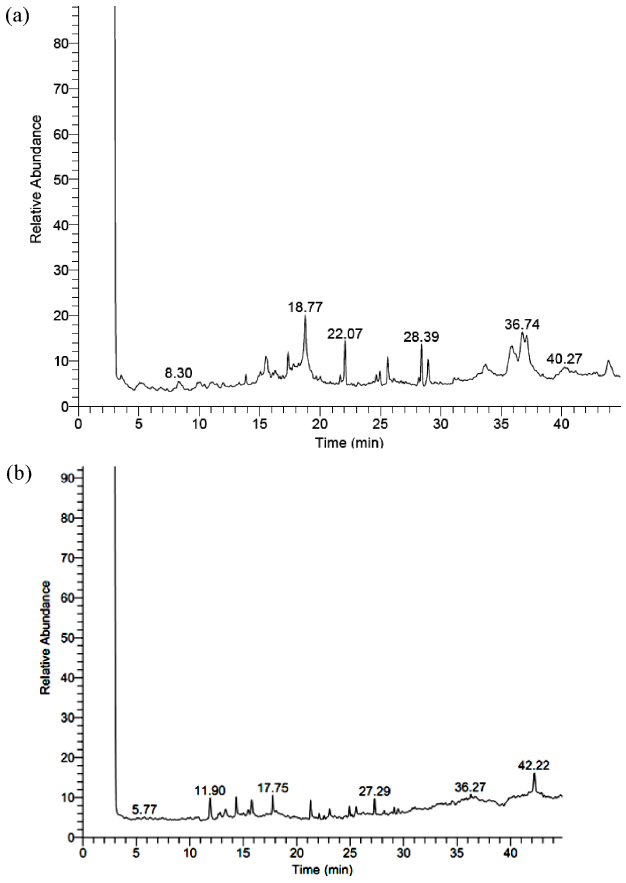

Fig. 6 – *GC-MS chromatogram of compounds residue extracted from a) spent wash treated using C. cladosporioides (first stage), b) spent wash treated using Phormidium valderianum (second stage)*

Conclusion

In the present investigation, anaerobically treated distillery spent wash was degraded and decolourized successfully by the two-stage sequential treatment using fungi (*C. cladosporioides*) and cyanobacteria (*P. valderianum*). Apart from degradation, major valuable products of 2,7-octadieniol acetate and 2-(1-decenyl)-tetrahydrofuran were obtained through the metabolic reaction in each stage of the treatment, which were analyzed and confirmed using GC-MS and HPLC.

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References

1. *Raghukumar, C., Mohandass, C., Kamat, S., Shailaja, M. S.*, Simultaneous detoxification and decolorization of molasses spent wash by the immobilized white-rot fungus Flavodon flavus isolated from a marine habitat, Enzyme Microb. Technol. **35** (2004) 197. doi: http://dx.doi.org/10.1016/j.enzmictec.2004.04.010
2. *Dahiya, J., Singh, D., Nigam, P.,* Decolorization of molasses wastewater by cells of Pseudomonas fluorescents on porous cellulose carrier, Bioresour. Technol. **78** (2001) 110 doi: http://dx.doi.org/10.1016/S0960-8524(00)00163-2
3. *Thakkar, A. P., Dhamanakar, V., Kapadnis, B.,* Biocatalytic decolourisation of molasses by *Phanerochaete chrysosporium*, Bioresour. Technol. **97** (2006) 1387. doi: http://dx.doi.org/10.1016/j.biortech.2005.07.005
4. *Pazouki, M., Najafpour, G., Hosein, M. R.,* Kinetic models of cell growth, substrate utilization and bio-decolorization of distillery wastewater by *Aspergillus fumigatus*, Afr. J. Biotech. **7** (2008) 1369.
5. *Kalavathi, D. F., Uma, L., Subramanian, G.,* Degradation and metabolization of the pigment-melanoidin in distillery effluent by the marine cyanobacterium Oscillatoria boryana BDU 92181, Enzyme Microb. Technol. **29** (2001) 246. doi: http://dx.doi.org/10.1016/S0141-0229(01)00383-0
6. *Ghosh, M., Ganguli, A., Tripathi, A. K.,* Treatment of anaerobically digested distillery spentwash in a two-stage bioreactor using *Pseudomonas putida* and *Aeromonas* sp, Process Biochem. **37** (2002) 857. doi: http://dx.doi.org/10.1016/S0032-9592(01)00281-3
7. *Krishna, P. R., Srivastava, S. N.,* Electrochemical degradation of distillery spent wash using catalytic anode: Factorial design of experiments, Chem. Eng. J. **146** (2009) 22. doi: http://dx.doi.org/10.1016/j.cej.2008.05.008
8. *Kaushik, G., Gopal, M., Thakur, I. S.*, Evaluation of performance and community dynamics of microorganisms during treatment of distillery spent wash in a three stage bioreactor, Bioresour. Technol. **101** (2010) 4296. doi: http://dx.doi.org/10.1016/j.biortech.2010.01.046
9. *Amit, D., Nagaraja, T. P., Dhiraj, S.,* Studies on coupled biological and photochemical treatment of soda pulp bleaching effluents from agro residue based pulp and paper mill, J. Chem. Technol. Biotechnol. **86** (2011) 1505.
10. *Ravikumar, R., Vasanthi, N. S., Saravanan, K.,* Single factorial experimental design for decolorizing anaerobically treated distillery spent wash using *Cladosporium cladosporioides*, Int. J. Environ. Sci. Technol. **8** (2011) 97. doi: http://dx.doi.org/10.1007/BF03326199
11. *Ravikumar, R.*, Effect of transport phenomena of *Cladosporium cladosporioides* on decolorization and chemical oxygen demand of distillery spent wash, Int. J. Environ. Sci. Technol. **12** (2015) 1581. doi: http://dx.doi.org/10.1007/s13762-014-0520-4
12. *Chavan, M. N., Kulkarani, M. V., Zope, V. P., Mahulikar, P. P.,* Microbial degradation of melanoidins in distillery spent wash by an indigenous isolate, Ind. J. Biotechnol. **5** (2006) 416.
13. *Ramya, M., Anusha, B., Kalavathy, S., Devilaksmi, S.,* Biodecolorization and biodegradation of reactive blue by *Aspergillus* sp., Afr. J. Biotechnol. **6** (2007) 1441.
14. *Sharma, A., Sharma, S., Gupta, M. N.,* Purification of alkaline phosphatase chicken intestine by three phase partitioning and use of phenyl-Sepharose 6B in the batch mode, Bioseparation **9** (2000) 155. doi: http://dx.doi.org/10.1023/A:1008195729472

15. *Dhananjay, S. K., Muliman, V. H.,* Three-phase partitioning of galactosidase from fermented media of *Aspergillus oryzae* and comparison with conventional purification techniques, J. Ind. Microbiol. Biotechnol. **36** (2009) 123. doi: http://dx.doi.org/10.1007/s10295-008-0479-6
16. *Jhadav, A., Vamsi, K. K., Khairnar, Y., Boraste, A., Gupta, N., Trivedi, S., Patil, P., Gupta, G., Gupta, M., Mujapara, A. K., Joshi, B., Mishra, D.,* Optimization of production and partial purification of laccase by *Phanerochaete chrysosporium* using submerged fermentation, J. Microbiol. Res. **1** (2009) 09.
17. *Buddolla, V., Subhosh, C. M., Pallavi, H., Rajasekhar, R. B.,* Screening and assessment of laccase producing fungi isolated from different environmental samples, Afr. J. Biotechnol **7** (2008) 1129.
18. *Raj, A., Reddy, M. M. K., Chandra, R.,* Identification of low molecular weight aromatic compounds by gas chromatography–mass spectrometry (GC–MS) from kraft lignin degradation by three *Bacillus* sp, Int. Biodeterior. Biodegradation. **59** (2007) 292. doi: http://dx.doi.org/10.1016/j.ibiod.2006.09.006
19. *Ravikumar, R., Monash, P., Derek, J. C. Chan, Saravanan, K.,* Microbial decolorization of biomethanate distillery spent wash using *Aspergillus nidulans*, Asian Jr. Microbiol. Biotech. Env. Sc. **2** (2010) 337.
20. *Watanabe, Y., Sugi, R., Tanaka, Y., Hayashida, S.*, Enzymatic decolorization of melanoidin by *Coriolus* sp., Agric. Biol. Chem. **46** (1982) 1623. doi: http://dx.doi.org/10.1271/bbb1961.46.1623
21. *Benito, G., Miranda, M. P., Rodriguez de los Santos, D.,* Decolorisation of wastewater from an alcoholic fermentation process with *Trametes versicolor*, Bioresour. Technol. **61** (1997) 33. doi: http://dx.doi.org/10.1016/S0960-8524(97)84695-0
22. *Kumar, L., Rathose, V. S., Srivastava, H. S.,* ^{14}C-[lignin]-lignocellulose biodegradation by bacteria isolated from polluted soil, Ind. J. Exp. Biol. **39** (2001) 584.
23. *Daneshvar, N., Ayazloo, M., Khatae, A. R., Pourhassan, M.,* Biological decolorization of dye solution containing malachite green by microalgae *Cosmarium* sp, Bioresour. Technol. **98** (2007) 1. doi: http://dx.doi.org/10.1016/j.biortech.2006.05.025
24. *Kumar, P., Chandra, R.*, Decolourisation and detoxification of synthetic molasses melanoidins by individual and mixed cultures of *Bacillus* spp. Bioresour. Technol. **7** (2006) 2096. doi: http://dx.doi.org/10.1016/j.biortech.2005.10.012
25. *Mohandass, R., Anusha, B., Kalavathy, S., Devilaksmi, S.,* Biodecolorization and biodegradation of reactive blue by *Aspergillus* sp, Afr. J. Biotechnol **6** (2007) 1441.
26. *Chandra, R., Bharagava, R. N., Rai, V.*, Melanoidins as major colourant in sugarcane molasses based distillery effluent and its degradation, Bioresour. Technol. **99** (2008) 4648. doi: http://dx.doi.org/10.1016/j.biortech.2007.09.057
27. *Ravikumar, R., Renuka, K., Sindhu, V., Malarmathi, K. B.,* Response surface methodology and artificial neural network for modeling and optimization of distillery spent wash treatment using *Phormidium valderianum* BDU 140441, Pol. J. Environ. Stud. **22** (2013) 20.