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Biodiesel, bioflocculant and biosorbent from the fungal *Curvularia sp.* strain DFH1

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ABSTRACT

Fungi are a good worker in many environmental duties, where the use of an organism to produce more than one product in the same medium is profitable. Exopolymer, total lipids and mycelium mass, etc... are the scope of different separate studies. In the present work a fungal Curvularia sp. strain DFH1 can act as oleaginous where it contain about 26% of its dry weight as lipids with a Fatty acid profile of hexadecanoic, octadecenoic, octadecanoic and erucic. Which have a valuable economic impact or it can act as a feed stock for biodiesel production, also and at the same time produce an exopolymer which act as a good powerful bioflocculants giving flocculation activity reached to 95%, The FTIR spectra of bioflocculant showed the presence of carboxyl, hydroxyl, amide and amino groups. And the cell debris functions as heavy metal absorbent biomaterial, where about 85% Cd(II) and 15% Zn(II) were removed by cell debris as biosorbent. So the purpose of this paper is to view the various attributes of the fungal isolate and explores the possibility of utilizing them in different ways in a manner of zero waste process.

Key words: Biodiesel; Bioflocculant; Biosorbent; Curvularia

INTRODUCTION

Fungi are used in many ancient and modern biotechnological processes, such as the production of enzymes, vitamins, polysaccharides, pigments and lipids. Some of these products are produced commercially while others are potentially valuable in biotechnology. In addition, metabolically inactive fungal dead biomass due to their unique chemical composition sequesters metal ions and metal complexes from solutions. Today, fungal biotechnology is a major participant in global industry.

Some of fungi are reported to be oleaginous which can accumulate significant amounts of lipids [1, 2]. Fungi metabolically transform the external carbon into carbohydrate or hydrocarbon and then to lipids. Lipids are considered to be important storage compounds in the form of triacylglycerols (TAG). If the lipid content in the cell exceeds 20%, then microorganism can be called as oleaginous. Therefore, oleaginous filamentous fungi are suggested as a favorable feedstock for a sustainable biodiesel industry[3, 4].

Lipid accumulation in oleaginous molds has been demonstrated to occur when a nitrogen source in the medium becomes limited and the carbon source is present in excess, then the growth rate slows down and the synthesis of

proteins and nucleic acids tends to cease. What is mentioned previously that in non-oleaginous species, the carbon excess remains unutilized or is converted into storage polysaccharides, while, in oleaginous species, it is preferentially channeled toward lipid synthesis, leading to the accumulation of TAG within intracellular lipid bodies [5]. However in the present study the coproduction of lipids in addition to exopolymer which act as a good bioflocculant was observed.

Extracellular biopolymer was isolated and subjected for further bioflocculant experiments. It is now established that, in comparison with the widely used chemical flocculants, including aluminum sulfate, ferric chloride, and polyacrylamide that have been widely used, bioflocculants show more advantages because of their safety and biodegradability [6, 7]. So, screening for novel microorganisms which could produce bioflocculants with excellent flocculating activity, cost-effective production process and high polymer yield is one of the main targets of many research groups worldwide[8, 9].

It is known that filamentous fungi are used in several fermentation industries to produce varied compounds releasing thousands of tons of residual cell walls. To date, incineration is the main way of destroying this by-product. On the other hand, there is found highly metal-loaded waste water which produced from different industries. Existing treatments to purify these effluents are often less affordable than the taxes required to dispose of them in effluent [10]. Studies on the mechanism of removal of metal ions by microorganisms assessed that the cell wall is the primary site of metal ion accumulation [11, 12]. It has also been shown that the uptake of heavy metal cations is not mediated by metabolic processes, and can take place in dead as well as living cells. These findings offered the possibility of using dead waste mycelia for biosorption. Employment of such biosorbent would offer an attractive potential use of this waste material. Muzzarelli et al., have already reported success in this area[13].

In modern biotechnological processes, several products are often obtained simultaneously. In addition to lipids as a biodiesel feed stock and exopolymer as a good bioflocculant, fungal mycelium can be a source of chitin as a good heavy metal absorbent. Formation of three products in one biotechnological cycle would reduce their cost and make the method more profitable. The objectives of the present study are to test the ability of the filamentous fungal isolate for its lipids accumulation, secretion of a bioflocculant exopolymer and to combine the isolation of the main two products with obtaining cell wall as another target product for heavy metal removal.

MATERIALS AND METHODS

Sample collection and isolation of fungus

A water sample was collected from Al-Ibrahimia canal, Asyut, Egypt, in the depth of 10–30 cm, the samples were then stored at 4°C until use. 1 mL of the water sample was serially diluted to 10 fold by distilled sterile water and plated on yeast peptone dextrose agar plates (YPD) (in g/L: D-xylose 100, yeast extract 1.0, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.75, Na_2HPO_4 1.0, $CaCl_2.2H_2O$ 0.2, $FeCl_3$ 0.01, $ZnCl_2$ 0.01) with the initial pH 6 and supplemented with 50 mg/L Rose Bengal (4,5,6-Tetrachlorofluorescein) and 100 mg/L chloramphenicol. The plates were incubated at 30°C for 3 days. Pure cultures of fungi were isolated by cycles of re-plating on YPD agar plates.

Screening for oleaginous fungus

The procedure of prescreening for oleaginous fungus was performed on a 72 hours old slant of pure isolates. Directly, Fungal biomass was stored in dark with 0.5 mL PBS solution and 0.05 mL Nile-red solution (Nile red 25 µg Nile-red/ acetone 1000 mL) for 30 min then, a thin film was made on clean glass slide after washing the biomass with distilled water and kept for drying in the air. Thereafter, slide was observed and photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan). The fungal isolate designated DFH1 gave the highest red florescence signal was subjected for further analysis.

Identification of fungus

The isolated fungus DFH1 was identified to the genus level on the basis of morphological (color, texture appearance, and diameter of the colonies) and micro morphological characteristics on potato dextrose agar medium (showing the spore and mycelium shape), and the most updated keys for identifications.

Biomass production, lipid extraction and characterization

Strain DFH1was cultured in basal medium (in g/L: yeast extract 0.5, $MgSO_4.7H_2O$ 0.4, KH_2PO_4 2.0, $CaCl_2$ 0.5, $CuSO_4$ $5H_2O$ 0.05 and 5% glucose (w/v), with initial pH 6.0). Samples were withdrawn every 24 hours for seven days. The culture broth was centrifuged where; the supernatant was preserved to the next step. However, microbial cells were harvested and washed with distilled water three times, and then freeze dried at –50°C. Exacted weight was taken, and then total lipids were extracted from the dried biomass with chloroform: methanol, volume ratio of 2:1. Ultrasonication to favor cell membrane disruption during extraction was done. The mixture containing extracted lipids was separated from residual biomass by centrifugation and the solvent fraction was transferred to a new tube. Then, the residual of solvent was removed in a rotary evaporator followed by lyophilization to determine the ratio of extracted lipids in compare to the cell dry weight. Subsequently, transesterfication reaction was carried out using sulfuric acid as catalyst in flasks at following conditions: 30:1 molar ratio of methanol to lipids, 160 rpm, 5 h of reaction time, temperature at 55°C and 80% catalyst amount based on lipids weight. The reaction mixture was cooled and undisturbed until two layers were formed in a separating funnel. The upper layer (biodiesel) was separated with petroleum ether and the final biodiesel product was obtained by evaporating the ether from the solution.

The fatty acid methyl esters of biodiesel were analyzed by GC/MS. It was performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50–550; source at 230 °C and quadruple at 150 °C) in the EI mode with an HP-5ms capillary column (30 m ´ 0.25 mmi.d., 0.25 mm film thickness; J & W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The injection volume was 1 mL, with a split ratio of 50:1.

Bioflocculant from strain DFH1

To determine the ability of strain DFH1 to produce exo-biopolymer having the capability to be used as a bioflocculant; the slightly viscous broth obtained above after removing mycelium was precipitated by two volumes of cold ethanol. The precipitate obtained was re-dissolved in deionized water and re-precipitated by adding two volumes of cold ethanol with stirring. After several hours in refrigerator, the precipitate was collected, dissolved and precipitate like above, then the precipitate was washed with ethanol, air dried and lyophilized to be characterized.

After ensuring the presence of polymer in the broth, the flocculating activity of the biopolymer was measured by calculating the flocculating rate using the methods described elsewhere[9]. A mixture of synthetic clay suspension (5g/L) with a known volume of fungal broth in the presence of $CaCl_2$ was stirred with rapid mixing at 230 rpm for 2 min, followed by slow mixing at 80 rpm for 3 min using Laboratory Flocculator (Flocumatic 6PLAZAS/sample, Spain) and left standing for 3 min. A sample for optical densities (OD) measurement was withdrawn using automatic pipette from a height of 3 cm below the surface of clay suspension. Relying on the upper phase OD for clay suspension that was measured at 540 nm with a spectrophotometer (7230G, Shanghai, China) the flocculation efficiency of the isolated strain was screened. The flocculating activity was calculated according to the following equation:

Flocculation activity (%) = (a-b)/a*100

Where a and b are the supernatant optical densities (OD) of the control (clay suspension without any bioflocculant addition) and sample respectively, at 540 nm.

To characterize the extracted bioflocculant, total sugar content of the bioflocculant was determined by the phenol-sulfuric acid method using glucose as standard solution[14]. The protein content was measured by the Lowry-Folin method using bovine serum albumin as a standard. For FT-IR measurements, the absorption spectrum between 500 and 4,000/cm was measured for the dried biopolymer. Spectra were recorded using attenuated total reflectance (ATR) on a Bio-Rad FTS 6000 FT-IR spectrometer.

The fungal strain DFH1 as a biosorbent of heavy metals

Cadmium and Zinc solutions and standards were prepared using analytical grade anhydrous cadmium and Zinc (II) chloride salts ($CdCl_2$, $ZnCl_2$Acros organics, USA). All reagents were prepared in ultrapure water with resistance of 18.2 MΩ × cm. Fungal biosorbent was obtained as a cell residual from the mycelium of strain DFH1 cultured in basal medium as described above. The biosorbent was oven dried overnight at 60°C. It was then ground finely to

particles with size ≤ 150 µm. The biosorption experiment was conducted with 100 mL of 100 mg/L initial concentrations of cd (II) and Zn (II) solutions in 250 mL Erlenmeyer flask for 60 minutes of contact time at room temperature 26 ± 2°C with agitation at 150 rpm in shaker incubator. PH was adjusted to 6. After biosorption, samples were centrifuged at 1400 rpm for 20 minutes (1024, Kubota, Japan). The concentrations of metal ions were analyzed by using a graphite furnace atomic absorption spectrophotometer (GBC 932 AA, Australia). Each experiment was repeated three times and the results given are the average values.

RESULTS AND DISCUSSION

Identification of the fungal strain DFH1 and lipids detection

Morphological and micromorphological characteristics demonstrated that the isolated fungal strain DFH1 is belonging to the genus Curvularia. It is known that the genus Curvilaria is one of the most important members of family Dematiaeceae, the large and heterogenous group of moulds. The organisms are wide spread in environment and are found in soil and plant debris [15]. Where, strain DFH1 produces rapidly growing, woolly colonies on potato dextrose agar at 25°C. From the front, the color of the colony is white to pinkish gray initially and turns to olive brown or black as the colony matures. From the reverse, it is dark brown to black. Microscopic examination of strain DFH1 showed septate, slightly curved, brown conidia borne singly on a geniculate conidiophore (Fig. 1). So, the isolate DFH1are identified as Curvilaria and that is confirmed through the repeat of identification in the regional center of mycology, at Al-Azhar University, Cairo, Egypt.

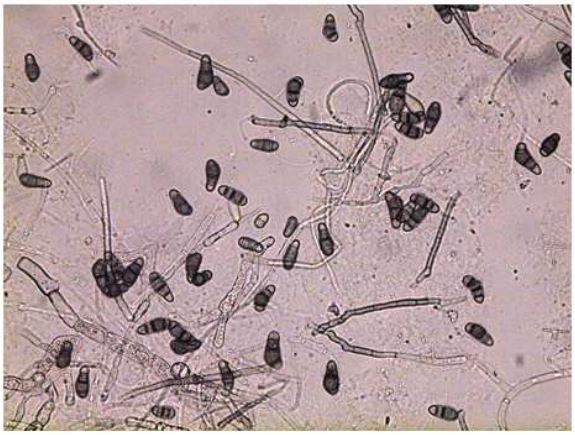

Figure 1: Morphological characteristics of the fungal strain DFH1 observed and photographed under light microscope

In addition, microscopic observation of the oleaginous fungi DFH1 has indicated the existence of lipid globules. However, direct observation under the microscope may not give a correct picture unless there is a specific method to observe the lipid particles. Several dyes, including Sudan black B [16], Luminor 490PT [17], and Nile red which is a marker of cytoplasmic lipid droplets in mammalian cells [18] were reported to determine the lipid content of cells, but Nile red seems preferable for the intracellular lipid determination. As shown in Fig. 2,strain DFH1exhibited strong red fluorescence signal and showed some organelles with different diameter stained with the red color, the isolate was visualized by light and fluorescence microscope[19].

Biodiesel production and characterization by strain DFH1

As shown in Figure (3) both cell dry weight and lipid yield of strain DFH1 were of almost in parallel during the seventh days of incubation. Strain DFH1 showed maximum lipid content of 26% of its dry weight which equal to 20 g/L (w/w) with maximum lipid yield of 5.2 g/L. Recently [20, 21]recorded similar pattern of performance with respect to biomass production and lipid yield with *Alternaria* sp. and *Aspergillus* sp., the lipid yield was found to be as high as 7.8 g/L and 3.1 g/L corresponding to the highest biomass production of 14.6 g/L and 13.6 g/L, respectively.

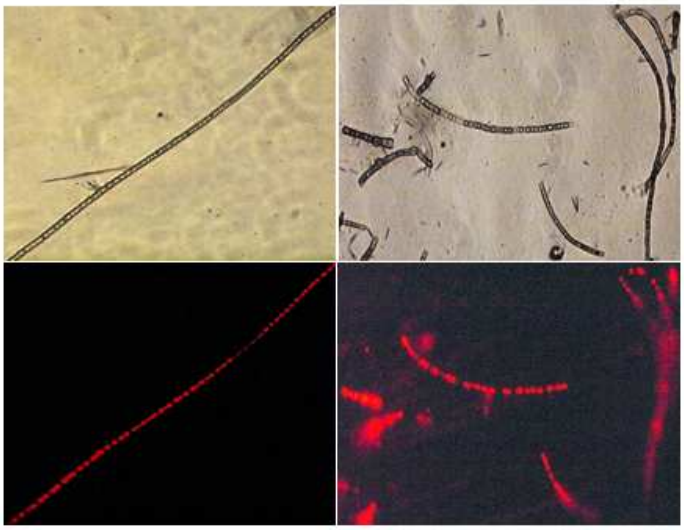

Figure2: Confirmation of lipid production in strain DFH1 using Nile-red staining assay

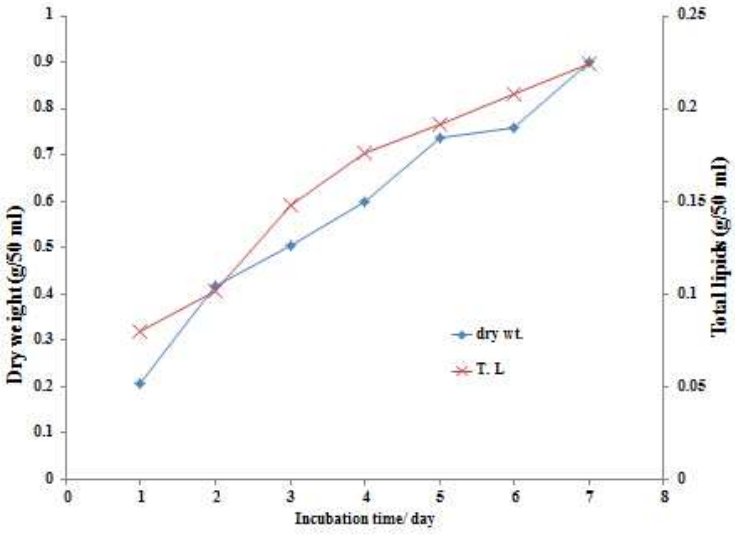

Figure3: Time course of both growth and lipid yield of strain DFH1 grown in basal medium containing 5% glucose

Furthermore, the fatty acid composition of strain DFH1 was analyzed by GC/MS in order to ascertain and compare their potential as biodiesel feedstock. The results presented in Table 1 showed the presence of the fatty acids hexadecanoic (29%), octadecenoic (54%), octadecanoic (12%) and erucic (3%), respectively. This result was in agreement with [22] where hexadecanoic acid plus stearic acid was the most abundant fatty acid isolated from the fungus *Geotrichum*. In addition[23]found that the lipid fraction of *C. japonica* VKMF was characteristic by octadecenoic acids reaching up to 50% of total fatty acids.

Table 1: Fatty acid composition of extracted total lipids from Curvilaria sp. strain DFH1 by GC/Mass

Fatty acid	Retention time	Peak Area	Height of peak	m/z	% of each one in the extracted total lipids
Hexadecanoic	37.926	40426224	6912983	74.00	29 %
11-Octadecenoic	43.519	89223443	9945100	55.00	54 %
Octadecanoic	44.200	16036250	2864652	74.00	12 %
Erucic	44.595	4252146	700501	55.00	3 %

Interestingly, the fatty acids profile of strain DFH1 indicates the presence of both saturated and unsaturated forms of fatty acids. Since the presence of unsaturated fatty acids only, show low oxidative stability especially under long periods in storage conditions above ambient temperatures with exposure to air and/or light [24]. Where, saturated forms tend to give more favorable properties of biodiesel. These include an increased cetane number (CN), decreased nitrogen oxides (NOx) emissions, shorter ignition delay time, and oxidation stability, but at the same time the saturated forms has also some disadvantage like relatively poor low-temperature flow properties. However, to ensure satisfactory in-use performance with respect to low temperature operability and oxidative stability, biodiesel should contain both long-chain saturated and poly-unsaturated fatty acids. In conclusion, these types of fatty acid produced by strain DFH1 have potential utilities for biodiesel production as have been documented for vegetable oils [25], oleaginous yeasts [26] and fungi [27].

Characterization of the exopolymer as a bioflocculants

In contrast to that observed in biodiesel production, the bioflocculation activity (FA %) of supernatant of lipid experiment was not in parallel with cell dry weight (DW) of strain DFH1 during the seventh days of incubation. As shown in Figure 4, cell dry weight was increased gradually by increasing the incubation period from 1 to 7 days as mentioned above. However, the FA% during this period increased and reached its maximum value of 95.6% after 72 h then a gradient decrease was observed till reach 0.0% after 7 days of incubation. This result indicated that strain DFH1 produces an extracellular bioflocculant. The same bioflocculant production pattern was reported with *Aspergillus parasiticus* [28] and *Aspergillus flavus* [29]. Accordingly, in order to produce bioflocculant with high flocculating activity, 72 h culturing time has been chosen. Concerning both bioflocculant production and DW profile, it is evident that the bioflocculant production was almost in parallel with cell growth till the first 72 hours studied which indicating that the bioflocculant was produced by biosynthesis during its growth, not by cell autolysis [30].

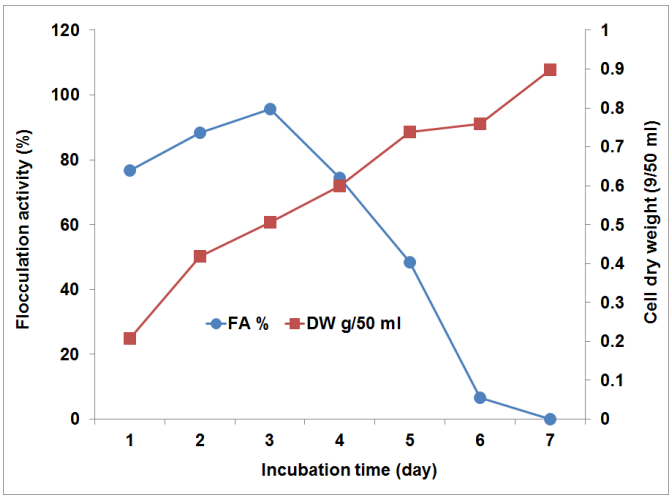

Figure 4: Time course of growth and bioflocculation activity of strain DFH1 (clay concentration =5g/L, agitation speed= 2min at 230rpm then 3min at 80 rpm, dosage of strain supernatant = 2.5ml/L, dosage of CaCl2 (3%) = 50ml/L clay supension pH=7)

The chemical analysis of the purified biopolymer produced by strain DFH1 demonstrates that it contains both protein and carbohydrate. However its major constituent is shown to be protein, where the proportions of the total sugar and protein content are 24% and 72% (w/w), respectively. As a method for confirming the chemical analyses and explores the relationship between the functional groups and the flocculation rate of the biopolymer, the FTIR spectrum (Figure 5) of the pure bioflocculant was analyzed. A broad stretching intense peak displayed around 3400 cm^{-1} can be assigned to the stretching vibration of OH and NH groups [31]. The peak at 2918 cm^{-1} is an indication of aliphatic C–H stretching. However, the peak at 1643 cm^{-1} may be due to the C=O stretching vibration in CONH group or NH_2 bending [32]. The strong peak observed a round 1063 cm^{-1} corresponding to the C-O stretching vibration in alcohols suggests the presence of OH group in the flocculant, and also it was generally known to be typical characteristics of all sugar derivatives. Consequently, the infrared spectrum indicates the presence of carboxyl and hydroxyl groups [33] which is in agreement with the chemical analysis results that the purified biopolymer contains both polysaccharide and protein. The protein and the polysaccharide contents of the purified

biopolymer may explain its stability toward all studied organic solvents (acetone, methanol, ethanol, chloroform, dimethylsulphoxide). However, it has a tendency to be soluble in water as well as both acidic and alkaline media.

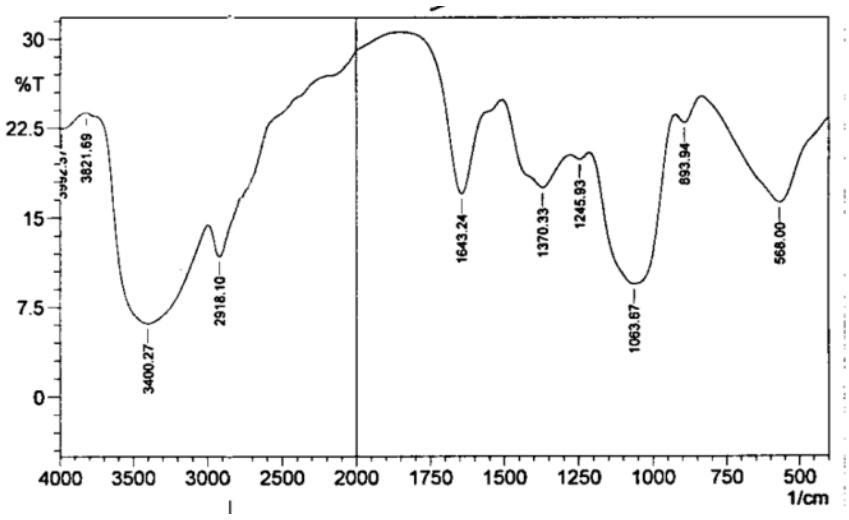

Figure 5: FT-IR spectra of the partially purified exo-biopolymer from strainDFH1

Usage of the fungal cell wall as a biosorbent

To mitigate the heavy metal pollution, many processes have been developed[34]. Biosorption is one of it, and it is the property of certain types of inactive, non-living microbial biomass to bind heavy metals even from very dilute aqueous solution. Cell wall structure of certain algae, fungi and bacteria was found to be responsible for this phenomenon [35].

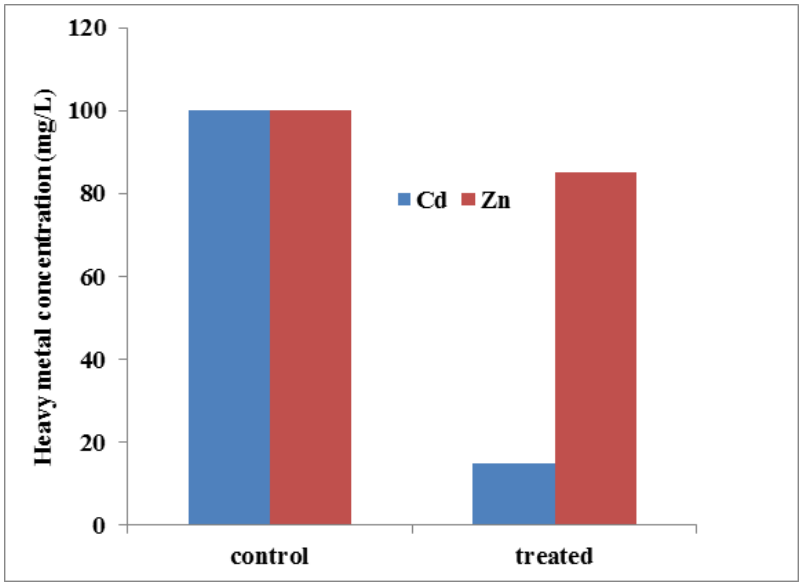

Figure 6: Biosorption of Cd(II) and Zn(II) onto the lyophilized fungal cell wall of the isolated fungus strain DFH1 from aqueous solutions at: pH, 6.0; temperature, 26 °C; and initial concentration of 100 mg/l

In the present study, biosorption of Cd(II)and Zn(II) onto the oven driedfungal cell wall debris derived from the above lipid extraction was investigated against artificial wastewater in the concentration of 100 mg/L. As shown in

Figure 6, results indicated that about 85 and 15% of metals is removed from solution after 1 hour contact time for cadmium and zinc, respectively. Previously [36]reported that *Aspergillus niger*, *Penicillium chrysogenum* and Claoicepspaspali have a removal capacity for zinc equal to 55, 8 and 12%, respectively. However, an exacted result for cadmium absorption was recorded by [37]. So, the use of waste-biomass in biosorption application could be helpful not only to the environment in solving the solid waste disposal problem, but also to the economy.

CONCLUSION

A fungus isolate was identified as Curvilaria sp. strain DFH1produced three products including lipids, exopolymer and mycelium mass on the same media. It was contain about 26% of its dry weight as lipids with a valuable economic impact as a feed stock for biodiesel production, also the exopolymer act as a good powerful bioflocculants for kaolin suspension, and the cell debris functions as heavy metal absorbent biomaterial. In light of these results it is either to grow the fungus for three days to take the advantage of the three products, but in this case the amount of lipid extracted will be reduced to the half, or it searches the optimal conditions for the extraction of the three products at large amounts and this needs to optimize some growth conditions and this is the research that are conducted in our labs now.

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