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Hemp Fibres as Novel Green Support Material for Immobilization of *Acidithiobacillus ferrooxidans*

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ABSTRACT

The suitability of hemp fibres as green support material for immobilization of *Acidithiobacillus ferrooxidans* cells in ferrous iron bio-oxidation process was investigated. A series of experiments were carried out in shake flasks and bioreactor for both cell immobilization and iron bio-oxidation. In flask experiments, five successive batch colonization cycles resulted in 6-fold increase in ferrous iron bio-oxidation rate. Evaluation of immobilized cells activity through two approaches as wet and dry storage demonstrated the higher activity of wet stored bacteria than dry ones after a period of two months. The dry stored bacteria lost almost all their activities after eight weeks. Whereas, the activity of wet stored samples was 30% at the same time. In bioreactor experiments, use of a packed-bed bioreactor with hemp fibres, the maximum ferrous iron bio-oxidation rate of 2.46 kg m^{-3} h^{-1} was obtained at dilution rate of 3.97 h^{-1}, and retention time of 0.25 h. Our experimental results could pave a new platform for the application of hemp fibre as an inexpensive and environmentally friendly support material in bacterial cell immobilization, especially in ferrous iron bio-oxidation process using acidophilic bacteria.

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NOMENCLATURE

τ	Hydraulic retention time
R	Bio-oxidation rate
D	Dilution rate

1. INTRODUCTION

Biological oxidation of ferrous iron to ferric iron is substantially promoted by *Acidithiobacillus ferrooxidans* catalytic activity [1]. For growth and maintenance, this chemolithoautotrophic bacterium uses carbon dioxide as carbon source, and obtains energy via ferrous iron bio-oxidation process [2]. The overall reaction of biological iron bio-oxidation is described by Equation (1) [3]:

$$2Fe^{2+} + ½\ O_2 + 2H^+ \xrightarrow{A.ferrooxidanse} 2Fe^{3+} + H_2O \quad (1)$$

Treatment of acid mine-drainage [4], removal of H_2S from sour gases [5], and bioleaching [6, 7] have been known as the main applications of biological ferrous iron oxidation. However, the industrial application of this process is restricted to low bio-oxidation reaction rate and also bacterial cells washout in high bioreactors flow rates. Increasing the bioreactors residence time may solve these problems; but it needed bioreactors with high working volumes which is not cost-effective [8].

Immobilization technique supposed to be a feasible method to overcome the above mentioned drawbacks. Use of immobilized bacterial cell in or on support materials has advantages over suspended cell cultures such as accessibility to high biomass density inside the bioreactor, reducing the bioreactor volume, and the possibility of bioreactor operation at high feed flow rates [9].

Up to now, various matrices have been used for immobilization process in ferrous iron bio-oxidation via entrapment or surface adhesion methods. The commonly used matrices were polyurethane foam [9-11], activated carbon [3, 12], and complex of poly (vinyl alcohol) and sodium alginate [13]. Other includes refractory clay tiles

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[14], and cotton gauze [7]. However, industrial application of immobilization technique is restricted to the acidic pH of *A. ferrooxidans* medium (~ pH 2). In addition, for bacteria immobilization, biodegradability, biocompatibility, and nontoxicity are critical points from environmental and economic points of view.

The objective of present research was to evaluate the potential of hemp fibres as green material for immobilization of *A. ferrooxidans* cells. One of the most valuable parts of the hemp plant (*Cannabis Sativ L.*) is fibre (bast) which grows as a stalk from the ground. In comparison with other natural fibres, hemp fibres have specific mechanical properties such as high stiffness and strength [15]. Moreover, biocompatibility, very straight forward preparation process, and being cost-effective are distinct advantages of this novel support material over conventional synthetic supports which were used for cell immobilization.

To the authors' best of knowledge, the application of hemp fibres for bacterial cell immobilization and its application in ferrous iron bio-oxidation process has not been reported. Herein, the performance of hemp fibres in *A. ferrooxidans* immobilization was investigated in both shake flask and packed-bed bioreactor. Also, the activity of the immobilized cells on hemp fibres was studied with respect to time. Finally, by applying different flow rates of fresh medium, the kinetic of continuous ferrous iron bio-oxidation in a packed bioreactor with hemp fibres was investigated.

2. MATERIALS

2. 1. Microorganism and Cultivation *Acidithiobacillus ferrooxidans* (PTCC 1646) was purchased from Iranian Research Organization for Science & Technology (IROST). The basal salt medium consisted of (in kg m^{-3}) $(NH_4)_2SO_4$, 0.4; $MgSO_4{\cdot}7H_2O$, 0.4; K_2HPO_4, 0.4 [11], devoid of ferrous iron. The initial ferrous iron concentration for cell growth, immobilization, and bioreactor continuous operation were 6.7, 5, and 1 kg m^{-3}, respectively. All basal salts were acquired from Merck, Germany. Throughout the study, the initial pH was adjusted to 1.6 by addition of concentrated H_2SO_4. All other reagents were of analytical grade, which were provided from commercial sources.

2. 2. Immobilization Procedure Creamy white hemp fibres which were sold as a washcloth were acquired from pharmacies in southern part of Iran. No pretreatment procedure was employed; just the hemp fibres were washed with distilled water and autoclaved.

For the immobilization, *A. ferrooxidans* cells were stabilized on hemp fibres via repeated batch colonization. In this process, the immobilized bacteria on the support material at the first cycle were considered as inoculum for the consequent cycles. A typical procedure was as follows: 7 g of hemp fibres were placed in 250 cm^3 flask with 50 cm^3 growth medium, followed by addition of ferrous iron at concentration of 5 kg m^{-3}. Then, 10% (v/v) of 45 h fully grew *A. ferrooxidans* culture was inoculated. The flask was incubated at 30 °C and 180 rpm. Upon consumption of 80% ferrous iron as the main substrate, the spent medium was replaced with the fresh medium without any bacteria inoculation. In order to remove precipitates and loosely attached cells, before transferring the fibres into fresh medium, hemp fibres were washed several times with acidic distilled water (pH of 2) [10]. This draw-and-fill process using fresh medium was continued up to the saturation of support material with bacterial cells.

2. 3. Storage of Immobilized Cells The activity of immobilized *A.ferrooxidans* cells with time was investigated based on two methods. One was storage at wet condition, in this method the samples were washed using acidic distilled water and then were suspended in fresh Fe (II)- deprived medium. In the other method, after washing with acidic distilled water, the supports were dried in the open air and packed in plastic. Both dry and wet prepared samples were stored at 4°C. In order to evaluate the activities of bacterial cells after storage, samples were taken out at the end of the first, second, fourth, and eighth week of storage. The cells were incubated in fresh medium in the same way as before storage [10]. The bacterial residual activity was defined as the ratio of time required by fresh immobilized support to that required by the stored support.

2. 4. Operation and Start-up of Bioreactor Continuous ferrous iron bio-oxidation was investigated in a packed bioreactor with hemp fibres. The main parts of the bioreactor consisted of a glass column with an inner diameter of 0.03 m, total height of 0.8 m, and a working height of 0.5 m. Fresh medium was introduced to the bottom of the bioreactor using a peristaltic pump. The bioreactor was aerated from the bottom of the column at the aeration rate of 500 cm3 min-1. The top part of the column incorporated outlets for the effluent stream and off-gas. A circulatory water jacket was employed for maintaining the temperature inside the bioreactor at 30 °C.

The bioreactor was operated in two stages: the start-up stage or biofilm formation stage and continuous ferrous iron bio-oxidation stage. In biofilm formation stage, 3 g of hemp fibres were placed inside a basket and settled in the column. The working volume of the bioreactor was filled with the medium at ferrous iron concentration of 5 kg m^{-3} and 10% *A. ferrooxidans* inoculation. Following this procedure, four successive batch cultures were operated without any additional inoculation. To prevent the formation of iron precipitate

(mainly jarosite) within the bioreactor [16], the successive draw-and-fill batches were quenched up to 80-90% bio-oxidation. For biofilm formation, a continuous recycling flow mode of operation was started via passing volumetric three-fold of bioreactor working volume of fresh medium at flow rate of 141.6 $cm^3 h^{-1}$ and ferrous iron concentration of 5 kg m^{-3}, without any additional inoculum. This stage lasted 34 h to achieve 80-90% ferrous iron conversion.

In the second stage, continuous feeding of fresh medium was initiated. This stage started at the same liquid flow rate that was used in the continuous-recycling mode. The liquid flow rate was increased stepwise when ferrous iron concentration altered by less than 5% (steady-state condition). Several liquid flow rates such as 141.6, 285, 507, and 1191 $cm^3 h^{-1}$ were evaluated during continuous operation. The ferrous iron concentration, pH, and cell concentrations were monitored by taking daily samples from the effluent of bioreactor at three times.

2. 5. Analytical Methods The free cell concentration was directly counted using a Neubauer chamber (haemocytometer) of 0.1 mm depth and 1/400 mm^2 area. Ferrous iron concentration was determined by a colorimetric procedure using 1,10-phenanthroline method [11]. Scanning Electron Microscopy (SEM) (model MIRA III, TESCAN, Czech Republic) analysis was carried out for monitoring the surface of the hemp fibres.

3. RESULTS AND DISCUSSION

3. 1. Cell Immobilization on Hemp Fibres The structure of fresh hemp fibres before cell immobilization is shown in Figure 1a.

Throughout the immobilization process, *A. ferrooxidans* cells were stabilized on hemp fibres via adhesion on the support surface. After conversion of ferrous iron to ferric iron using catalytic activity of immobilized bacteria, the medium color changed to red color which confirms successfully iron bio-oxidation (Figure 1b).

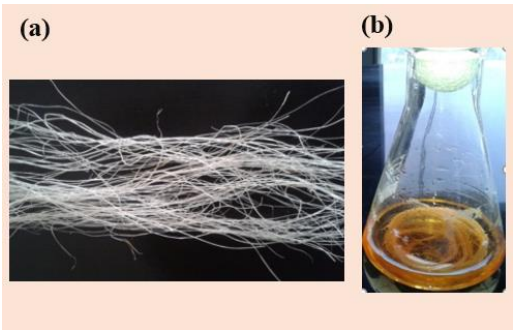

Figure 1. (a) structure of fresh hemp fibres; (b) iron bio-oxidation by immobilized cells on hemp fibers

Figure 2a, shows the final free cell density at the end of each immobilization cycle. After the second cycle of colonization, the number of immobilized bacteria on hemp fibres increased; thereby, the rate of ferrous iron bio-oxidation increased. According to obtained results, the required time for each immobilization cycle (consumption of 80% ferrous iron) reduced from 34 h to 21, 14, 10.5, and 6 h, in the 1st, 2nd, 3rd, 4th, and 5th cycle, respectively. In fact, approximately 6-fold increase in ferrous iron bio-oxidation rate was observed after 5 successive immobilization cycles (Figure 2b). This behaviour can be attributed to the development of biofilm formation on the support; which led to an increase in ferrous iron bio-oxidation rate and consequently reduction in immobilization time [10]. It is also interesting to note that, the final concentration of free cells in the medium started to increase after the fourth cycle, which means the hemp fibres were saturated with bacterial cells at the end of the mentioned cycle.

Morphology of hemp fibres before and after cell immobilization was monitored using SEM image analysis. As shown in Figure 3a, the surface of fresh

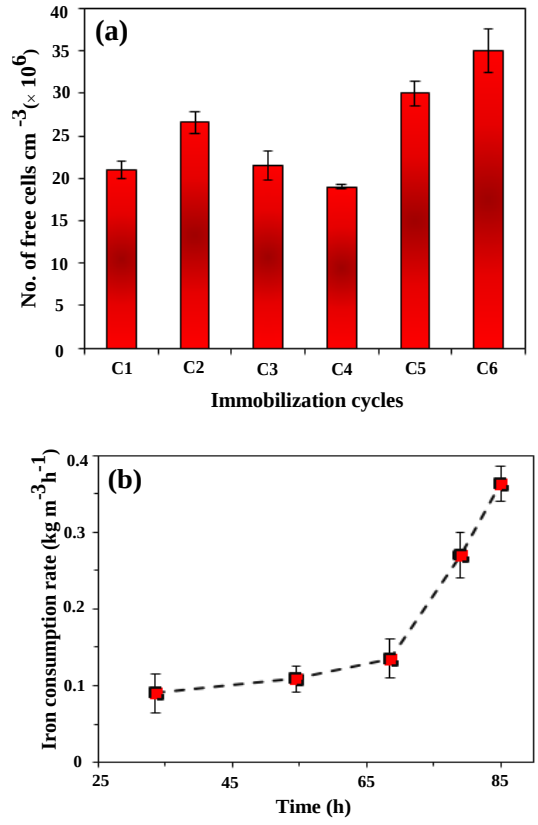

Figure 2. Immobilization of *A. ferrooxidans* cells on hemp fibres (a) free cell concentration and (b) ferrous iron consumption rate at the end of each immobilization cycle

hemp fibres has many deep grooves which provide the opportunity for bacterial cell immobilization. From Figure 3b it is clear that the structure of hemp fibre was not reformed after immobilization under acidic medium pH of 1.6. Figure 3c, confirms the attachment of *A. ferrooxidans* cells on the hemp fibres.

3. 2. Activity of Immobilized Cells after Storage

According to Figure 4, the activity of stored bacteria under both dry and wet conditions remained almost intact after one-week storage at 4°C. For wet stored samples, from the second to eighth week, gradually decline in the activity of cells was observed. As such, the required time for the prevalence of ferrous iron bio-oxidation increased from 15.3 h at the end of the first cycle to 16.87, 23.1, and 52 h at the end of the second, fourth, and eighth week of wet storage, respectively. In the case of dry storage, the activity of bacterial cells was rapidly decreased from the second up to the eighth week. At the end of the eighth week, bacteria that were stored dry lose almost 90% of their activities, wherease in wet storage, bacteria lose 70% of their activities.

Generally, throughout all the storage times, the bacteria activity was higher under wet storage condition in compare with dry stored samples. A similar phenomenon was observed by Jaisankar et al. [10], the wet stored (suspended in spent medium) *A. ferrooxidans* cells which were immobilized in polyurethane foam, retained almost 40% their activities after the thirteenth week. On the contrary, the activities of dry stored bacteria dropped up to 16% at the end of the tenth week. This behavior can be explained by the fact that the wet stored bacteria can use carbon dioxide or other nutrients that presence in spent medium or air for their maintenance; while in dry storage, accessibility to any sources of energy is limited [10]. Nevertheless, at 28 h time for complete ferrous iron bio-oxidation after 8 weeks storage of cell-immobilized hemp fibres at wet conditions is preferable to 48 h time of free cells ferrous iron bio-oxidation.

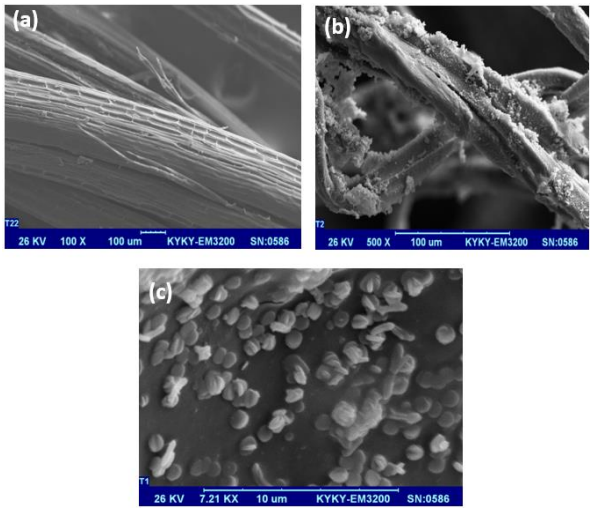

Figure 3. SEM images of hemp fibres (a) before and (b) & (c) after bacterial cell immobilization

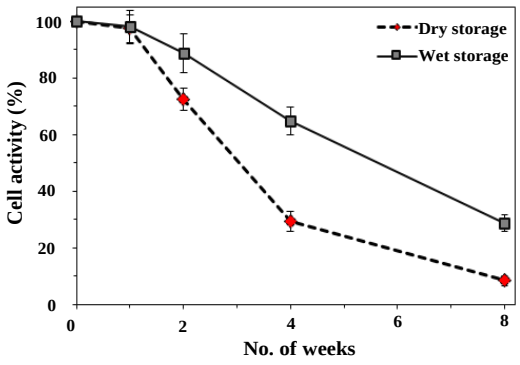

Figure 4. Activity of immobilized bacterial cells on hemp fibres under different storage conditions as a function of time

3. 3. Continuous Bio-oxidation

The packed-bed bioreactor with hemp fibres was started continuous operation after biofilm formation. Schematic illustration of the bioreactor is shown in Figure 5.

As shown in Figure 6, four different liquid flow rates were used during 306 h of bioreactor continuous operation. At steady-state condition (which lasted 2-3 days), the liquid flow rate was increased from 141.6 cm^3 h^{-1} to 285, 507, and 1191 cm^3 h^{-1}; thereby, hydraulic retention time (τ) decreased from 2.12 h to 1.05, 0.59, and 0.25 h, respectively.

According to Table 1, the maximum ferrous iron bio-oxidation rate (R) of 2.46 kg m^{-3} h^{-1} was obtained at the highest dilution rate (D) with biological oxidation efficiency of 70.2%. A comparable result was reported by Koseoglu-Imer and Keskinler [11], which have employed sulfonated poly (styrene–divinylbenzene) copolymer with granular activated carbon for

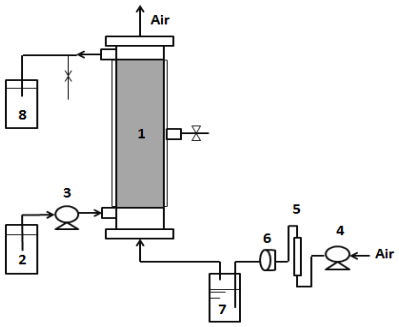

Figure 5. Schematic of bioreactor: (1) packed bed column, (2) fresh medium, (3) peristaltic pump, (4) air pump, (5) gas flow meter, (6) filter, (7) humidifier, (8) effluent culture

immobilization of *A. ferrooxidans*. Use of 2 kg m^{-3} ferrous iron as main substrate, the maximum iron oxidation rate of 0.77 kg $m^{-3}h^{-1}$ was reported at the hydraulic retention time of 2.6 h.

It was found that increasing feed flow rate led to an increase in ferrous iron bio-oxidation rate. In contrast, the ferrous iron conversion to ferric iron reduced. As shown in Figure 6, the highest iron conversion (91.35%) was obtained at the lowest liquid flow rate of 141.6 $cm^3 h^{-1}$. That was more, at the beginning of switching to the consecutive liquid flow rates, the iron conversion was reduced; which could be related to the bacterial cells wash-out. Therefore, due to the fixation of biomass on the support material, the ferrous iron conversion was enhanced at the end of each liquid flow rate. Koseoglu-Imer and Keskinler [11] also reported that at the start-up of their experiment (or when *A. ferrooxidans* cells were in the lag phase), the use of high liquid flow rates led to a decrease in ferrous iron conversion because of wash-out phenomena. After a period of time and formation of ferric iron precipitates on support material (sulfonated poly (styrene-divinylbenzene) copolymer with granular activated carbon), an increase in the number of the immobilized bacterial cell was observed.

Although precipitate is known as an unwanted phenomenon that restricts bacterial accessibility to nutrient because of mass transfer limitations [16]. The role of iron precipitate in the continuous operation of bioreactor for immobilization of bacterial cells is critical [17]. Further experiments are required to evaluate continuous bio-oxidation using hemp fibres immobilized cell at high concentration of ferrous iron in the bioreactor input.

TABLE 1. The kinetic parameters of continuous bioreactor operation

Flow Rate (cm^3h^{-1})	Kinetic Rameters		R (kg $m^{-3}h^{-1}$)
	D (h^{-1})	τ (h)	
141.6	0.472	2.12	0.43
285	0.95	1.05	0.84
507	1.69	0.59	1.4
1191	3.97	0.25	2.46

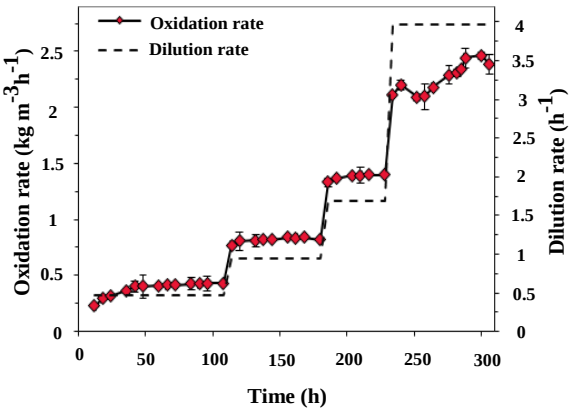

Figure 6. Ferrous iron bio-oxidation rate with time as a function of dilution rate at 30 °C

4. CONCLUSIONS

Biological oxidation of ferrous iron to ferric iron was investigated using immobilization technique. The experiments were focused mainly on the introduction of hemp fibre as a new support for bacterial cell immobilization. The results presented in this paper demonstrate great potential of hemp fibres in providing an appropriate contact surface for the colonization of *A. ferrooxidans*. In addition, this biodegradable support was strong enough under acidic pH in continuous bioreactor operation and also after two months of storage. Overall, the special structure of this natural plant waste material provides a suitable surface for bacterial cells attachment and makes it an alternative candidate for being used as support material in immobilization processes.

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چکیده

امکان استفاده از الیاف کنف در تثبیت باکتری اسید دوست *تیوباسیلوس فروکسیدان* با هدف کاربرد در اکسیداسیون بیولوژیکی آهن فروس مورد بررسی قرار گر فت. آزمایش‌های مربوط به تثبیت باکتری و نیز اکسیداسیون آهن، در دو مقیاس ارلن و بیوراکتور انجام گرفتند. بر اساس نتایج بدست آمده در مقیاس ارلن، پس از ٦ مرحله متوالی تثبیت باکتری، مقدار اکسیداسیون آهن شش برابر افزایش پیدا کرد. در ادامه تحقیق، باکتری تثبیت شده به مدت دو ماه طی دو روش مختلف خشک و مرطوب نگه داری شد. بر اساس نتایج بدست آمده، فعالیت باکتری ذخیره شده در حالت مرطوب بیشتر از حالت خشک بود. به طوریکه در حالت نگه داری در شرایط خشک تقریبا باکتری تمام فعالیت خود را در پایان هفته هشتم از دست داد. در مقابل، پس از گذشت زمان هشت هفته، باکتری نگه داشته شده در شرایط مرطوب، ۳۰ درصد فعالیت خود را حفظ کرده بود. در مقیاس بیوراکتور، بیشترین شدت اکسیداسیون $2.46\ kg\ m^{-3}\ h^{-1}$ در شدت رقیق سازی $3.97\ h^{-1}$ و زمان اقامت ۰.۲ ساعت با استفاده از راکتور پر شده با الیاف کنف بدست آمد. براساس نتایج بدست آمده در این تحقیق، الیاف کنف مقاومت خوبی تحت شرایط pH اسیدی محیط کشت باکتری *تیوباسیلوس فروکسیدان* از خود نشان دادند. بر این اساس می‌توان از این حامل ارزان و زیست سازگار برای تحقیق‌های آینده جهت تثبیت سلول‌های باکتریایی تحت شرایط خاص pH محیط های کشت استفاده کرد.

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