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Characterization of Bacillus cereus Bacteria Isolated from Egyptian Iron Ore Surface

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ABSTRACT
In mineral bio-beneficiation, it is very important to understand the microbial surface characteristics and its behaviour onto the mineral surface. *Bacillus cereus* bacterium has never been used before as a bio-reagent for separation of different mineral systems. In this paper, complete characterization of such type of bacteria, isolated from Egyptian iron ore surface, including gram stain, growth curve, Biolog microbial identification, Zeta potential measurements, FTIR measurements, Scanning Electron Microscope, SEM as well as protein and polysaccharide analysis have been studied. The results confirmed that Bacillus cereus is a gram positive bacterium, rod shaped, smooth and circular with different types of by-products as polysaccharides, carboxylic acids and amino acids that gives an amphoteric behaviour on the cell surface. The results of zeta potential showed that the iso-electric point (IEP) of iron oxide (≈ 6.3) is significantly displaced to lower values (≈ 2.2) after treatment with the bacterial isolates which indicates the bacterial hydrophobic effect. This is in agreement with FTIR results which confirmed the formation of hydrogen bond between OH group (of the polysaccharides part) and/or the COOH group of both the polysaccharides or the protein fractions of the bacteria with the positively adsorption sites of hematite lead to a reduction in the zeta potential of its surface to be close from that of the bacteria itself. The results obtained showed a better affinity of *Bacillus cereus* to hematite mineral and could be used in separation of such mineral from its associated gangue minerals.

Introduction

The necessity to process and beneficiate ores with lower grades, which are more refractory and finely disseminated, has resulted in the conventional mineral processing and hydrometallurgical operations becoming inadequate and inefficient. Further, stringent environmental regulations have constrained the processing of ores. It thus becomes imperative to develop novel technologies for mineral processing and waste remediation. Recent developments in biotechnology hold promise to process such difficult-to-treat ores as well as to safeguard the environment. The growing importance of mineral bioprocessing can be gauged from the conferences exclusively dedicated to this topic [1-4]. An excellent overview of mineral bioprocessing has been compiled by Smith and Misra (1991b)[5]. Typical examples of the potential applications of microbes in mineral beneficiation [6, 7], flotation collectors [8] and flotation modifiers [9, 10]. These bacterial isolates act as bio-reagents and may induce hydrophobic properties once they can adhere selectively onto the mineral surface [11]. The micro-organism cell surface is conformed by functional groups like polymers, peptides, phospholipids, proteins and organic acids [12]. Those groups must adhere to the mineral surface directly and utilize cell surface associated or extracellular biopolymers to catalyse chemical reactions on the mineral surface [1, 13 and 14]. Like traditional reagents, the bacterial isolate interacts with the mineral surface and gives amphoteric characteristics to it, [15]. Application of bio-reagents as collectors involves several fundamental aspects: surface charge, presence of specific hydrophobic groups and polymers compounds which deeply affect their adhesion to the mineral surface [13, 16 and 17]. Bacillus cereus is frequently isolated from both the natural environment (soil and growing plants) and foods, including raw and pasteurized milk and milk products [18], dried products [19], pulses and cereals [20], spices [21], meat products, raw meat and meat product additives [22], fresh vegetables and ready-to-eat vegetable-based foods [23]. There is the first time in isolating *B. cereus* from mineral surface and studying its behaviour in mineral processing. In this paper, complete characterization of *B. cereus* bacterium isolated from iron oxide surface and its adsorption on the mineral surface has been studied.

Materials and Methods

Materials

Sample of single mineral of haematite (Fe_2O_3), was delivered from 'Wards' Company, USA. The purity (99.9 %) of the samples was confirmed using XRF. The –200 mesh fractions were used in adsorption studies. Analytical grade HCl and NaOH, from Aldrich, were used for pH regulations.

Methods

Bacterial Growing and Isolation

A suspension containing 0.5 gm of mineral sample in 10 ml distilled water was prepared. After that, 1 ml of suspension was taken and sprayed onto a nutrient agar plate surface then incubated for 24-48 hr at 30°C. The developed colonies were picked up and streaked on nutrient agar plates and incubated at 30°C for 24-48 hr. The final step was repeated several times until pure colonies have been obtained. Separate colonies were picked up, streaked on nutrient agar slopes, stored at 4°C and sub-cultured monthly [24].

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Growth Media

Two forms of nutrient media were used, solid form (nutrient agar) and liquid form (nutrient broth). Nutrient agar (NA) [25] including peptone, 5gm; beef extract, 3gm; sodium chloride, 8gm; agar, 12gm and distilled water, 1000ml. The constituents were dissolved with heating, adjusted to pH 6.8-7.0 and sterilized at 120^0C for 20 min.

Graphical Expression of Bacterial Growth

Bacterial growth was measured through inoculating the preserved bacterium into 100 ml nutrient broth and incubated overnight in 250 ml measuring flask after that optical density was measured to determine the start point. Then 1 ml of the previous culture was taken in 100 ml nutrient broth followed by measuring OD550 along time intervals of 30 min. OD550 was measured using "Perkin-Elmer" Spectrophotometer" model Lambda 3B. Growth curve was obtained by plotting the logarithm of OD550 versus time, [1, 24].

Preparation of Inoculum

A liquate of 350 ml was dispensed into 1 litre flask, then sterilized at 120^0C for 20 min. and after that inoculated with a loop full of the bacterial strain under test and incubated at 30^oC for 48 hr according to growth curve of three strains of bacteria under test.

The Gram Stain Technique

A loopful of tap water was placed on a slide; using a sterile cool loop transfer a small sample of the colony to the drop, and emulsify. The film was allowed to be air dried. The dried film was fixed by passing it briefly through the Bunsen flame two or three times without exposing the dried film directly to the flame. The slide should not be so hot as to be uncomfortable to the touch. The slide with crystal violet solution was flooded for up to one minute and was washed off briefly with tap water (not over 5 seconds) and drained. After that, the slide was flooded with Gram's Iodine solution, and be allowed to act (as a mordant) for about one minute followed by washing off with tap water and drainage. Excess water was removed from slide and blot, so that alcohol used for decolourization is not diluted. The slide was flooded with 95% alcohol for 10 seconds and washed off with tap water. (Smears that are excessively thick may require longer decolourization). This is the most sensitive and variable step of the procedure, and requires experience to know just how much to decolorize). The slide after that was drained, flooded with safranin solution and be allowed to counterstained for 30 seconds. Finally, the slide was washed off with tap water, drained and blotted dry with bibulous paper and didn't be rubbed. All slides of bacteria must be examined under the oil immersion lens.

BIOLOG Microbial Identification System

Bacteria identification was done using the BIOLOG GEN III Micro-plate microbial identification system. A pure culture was grown on BIOLOG recommended agar media and incubated at 30° C. Inoculums were prepared where the cell density was in the range of 90-98%T. precisely 100 µl of the cell suspension was transferred by multichannel pipette into the wells of BIOLOG micro-plate. The plates were incubated for 36 hours at 30° C into the Omni-Log incubator/reader. The BIOLOG micro-plate tests the ability of an organism to utilize or oxidize a pre-selected panel of 95 different carbon sources. The dye tetrazolium violet is used to indicate utilization of substrates. A panel of 95 different substrates gives a very distinctive and repeatable pattern of purple wells for each organism in which the manufacturers literature terms a "Metabolic Fingerprint". Finally; micro plate was read using BIOLOG's Microbial Identification Systems software through biology reader

Zeta Potential Measurements

A laser Zeta Meter 'Malvern Instruments Model Zeta Sizer 2000' was used for zeta potential measurements. 0.05 g of ground sample was placed in 50 ml double distilled water with definite concentration of the bacterial isolate at fixed ionic strength of 10^{-2} M NaCl. NaOH and HCL were used as pH modifiers. The suspension was conditioned for 60 minutes during which the pH was adjusted. After shaking, the equilibrium pH was recorded. It was then allowed to settle for 3 min, after which 10 ml of the supernatant was transferred into a standard cuvette for zeta potential measurement. Solution temperature was maintained at ($25^oC \pm 2$). Five measurements were taken and the average was reported as the measured zeta potential [1, 13].

Measuring Selectivity of Bacteria to Mineral Surface

A laser particle size analyzer (FRITSCH Model Analyst 22) was employed for measuring size analysis of single minerals before and after treatment with bacteria. Fixed volume 10 ml of Bacillus cereus was conditioned with one gram of each mineral for 60 minutes before recording the change in size distribution [1, 13].

FTIR Measurements

Infrared absorption spectra were recorded for haematite and bacterial isolate before and after interactions using Fourier transform infrared spectrometer (Model FT/IR 6300). After interaction with bacteria, the mineral samples were thoroughly washed using double distilled water and vacuum dried. The KBr pellet technique was used to record the spectra [1, 13].

HPLC Analysis

Samples were filtered through a 0.45 µm membrane (Smith et al., 1986). Analysis of the carbohydrate in the filtrate was performed by using HPLC, Shimadzu Class-VPV 5.03 (Kyoto, Japan) equipped with refractive index RID-10A Shimadzu detector, LC-16ADVP binary pump, DCou-14 A degasser and Shodex PL Hi-Plex Pb column (Sc 1011 No. H706081), Guard column Sc-Lc Shodex, and heater set at 80^o C. The mobile phase was water, and the flow rate was 1 ml / min. Standard solutions of individual sugars with analytical Glucose, fructose, Sucrose, and pectin, cellulose and hemicelluolse were prepared by diluting each analyzed sugar deionized water. Injection volume of each standard was 10 µL.

Amino Acid Analysis

Amino acid content was determined as described by Spackman et al. (1958)[26]. The analysis was performed in Central Service Unit, National Research Centre, Egypt, using LC3000 amino acid analyzer (Eppendorf-Biotronik, Germany). The technique was based on the separation of the amino acids using strong cation exchange chromatography followed by the ninhydrine colour reaction and photometric detection at 570 nm. Samples were hydrolyzed with 6 N HCl at 110 ^{o}C in Teflon capped vials for 24 h. After vacuum removal of HCl, the residues were dissolved in a lithium citrate buffer, pH 2.2. Twenty µl of the solution were loaded onto the cation exchange column (pre-equilibrated with the same buffer), then four lithium citrate buffers with pH values of 2.2, 2.8, 3.3 and 3.7, respectively, were successively applied to the column at flow rate 0.2 ml/min. The ninhydrine flow rate was 0.2 ml/min and pressure of 0-150 bar. The pressure of buffer was from 0 to 50 bar; and reaction temperature was 130 ^{O}C.

Results and Discussion

Bacterial Growing, Isolation and Identification

As shown in Fig.1, colonies of Bacillus cereus are circular, dull and opaque with a rough matted surface. Colony perimeters are irregular and represent the configuration of swarming from

the site of initial inoculation. Figure 2 indicated that the cells are gram positive, rod shaped.

Figure 1. Colony shape of B. Cereu

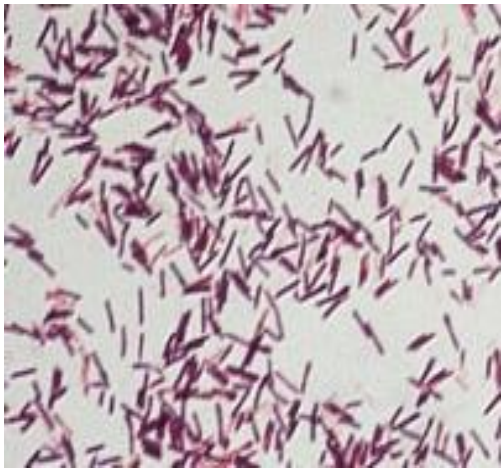

Figure 2. Gram stain of B. cereus (1000X)

Biolog ID Report

ID Result Species ID: Bacillus cereus
ID Comment
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	0.918	0.567	6.653	GP-RODSB	Bacillus cereus
2	0.072	0.042	7.572	GP-RODSB	Bacillus pseudomycoides
3	0.009	0.005	8.302	GP-RODSB	Bacillus weihenstephanensis
4	0.000	0.000	9.459	GP-RODSB	Bacillus mycoides

‹X›:Pos; ‹X—:Mismatched Pos; X:Neg; X ••:Mismatched Neg; (X):Borderline; -X:Less Than A1 well

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	‹ 250 ›	‹ 250 ›	‹ 250 ›	0	0	0	0	0	‹ 250 ›	‹ 250 ›	0
B	0	0	0	0	‹ 250-	‹ 250 ›	0	0	0	‹ 250 ›	‹ 250 ›	‹ 250 ›
C	‹ 250 ›	0	‹ 250 ›	0	0	0	0	0	‹ 250 ›	‹ 250 ›	0	0••
D	0	0	0	0	‹ 250 ›	‹ 250 ›	‹ 250 ›	0	‹ 250 ›	0	0	0
E	‹ 250 ›	0	0	0	(75)	(75)	‹ 250 ›	0	‹ 250 ›	0	‹ 250 ›	0
F	0	(75)	(75)	‹ 250 ›	‹ 250-	‹ 250 ›	0	0	0	0	0	0
G	0	(75)	0	(75)	0	0	0	0••	0	0	‹ 250 ›	‹ 250 ›
H	(75)	0	0	0	0	‹ 250 ›	(75)	(75)	(75)	‹ 250 ›	‹ 250 ›	‹ 250 ›

Graphical Expression of Bacterial Growth

As shown in Fig.3, The lag phases for bacteria are fairly short after which exponential growth takes place. The absorbance was taken as a measure of the maximum number of cells produced. The curve showed that the maximum number was obtained after 12 hours.

Surface Properties of Hematite and B. cereus

Generally, the value and sign of zeta potential depend upon pH of the medium, indicating that both H+ and OH- are potential determining ions for each mineral. The sign of zeta potential changes from positive to negative with changing the pH of the medium from the acidic to the alkaline range. The results, also, showed that the electronegativity of zeta potential increases gradually with increasing the pH. It is known that the zeta potential increases in magnitude with decreasing ionic strength of counter ions (NaCl), due to the increase in thickness of the diffuse layer, due to coulombic interactions which is a dominant role in adsorption process [27]. Measurement of zeta potential of Bacillus cereus alone as well as for hematite, in absence and presence of bacteria has been performed at constant ionic strength of 10^{-2} M NaCl over a wide pH range of 1-13, the obtained result is shown in Figure 4. For Bacillus cereus, the results clearly indicate that the curve is conducted at the definite ionic strength of electrolyte, intersect with pH axis at pH 2.49. This value is considered as isoelectric points (iep) for Bacillus cereus.

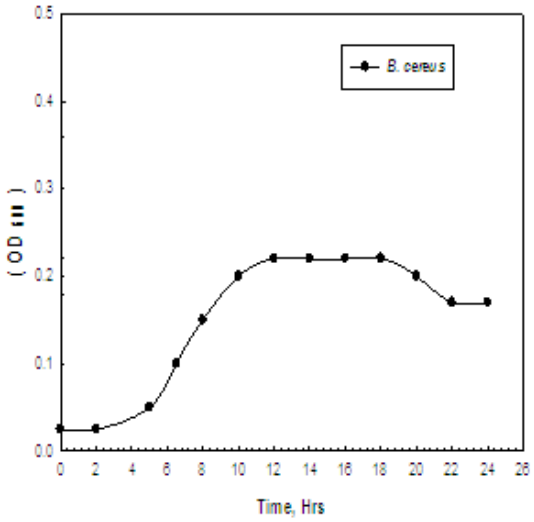

Figure 3. Growth Curve of Bacillus cereus

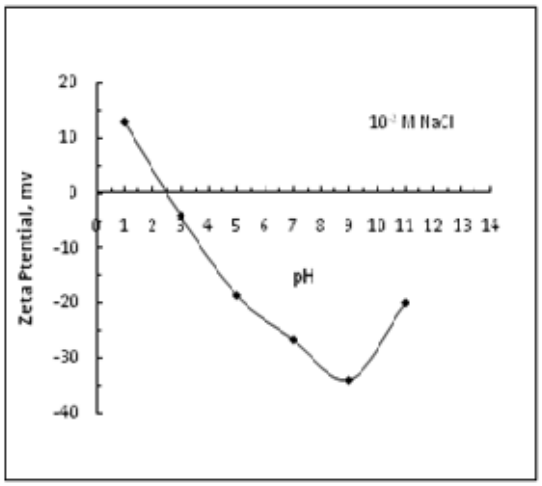

Figure 4. Zeta Potential of Bacillus *cereus*

Meanwhile, Figure 5 depicts the zeta potential of iron oxide as a function of pH before and after interaction with Bacillus cereus. The iso-electric point (IEP) of iron oxide corresponds to pH of about 6.3. Conditioning of iron oxide with Bacillus cereus resulted in a significant displacement for the IEP of iron oxide to that of Bacillus cereus, i.e., at pH of about 2.2. Interestingly, the values of zeta potential of iron oxide after interaction with bacterial isolate in the acidic pH range (pH 3 - 5) changed from positive to negative while those values in the alkaline pH range are, more or less, the same. These results indicated the hydrophobic character of mineral surface after treatment at this pH range with Bacillus cereus.

Measuring Selectivity of bacteria to Mineral Surface

The change in size distribution of iron oxide after its treatment with B. cereus was taken as a measure for the selectivity.

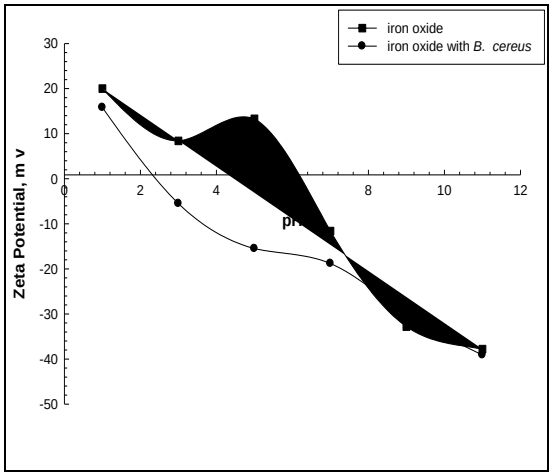

Figure 5. Zeta Potential of Iron Oxide treated with *B. Cereus*

Successful adsorption of B. cereus will cause, therefore, a degree of aggregation (or dispersion) for mineral particles leading to a change in their size distribution. As known, the larger the change in the size distribution the more selective the bacteria to the mineral surface. This technique was successfully used to screen different microorganisms for selective adhesion onto apatite or iron oxide surfaces [13, 28 and 29].

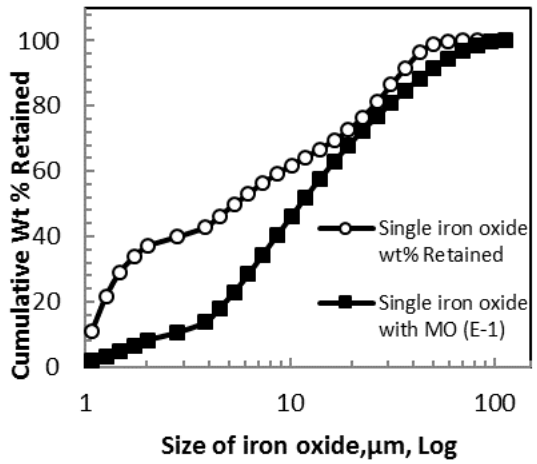

Figure 6. Size distribution of hematite with *B. Cereus*

FTIR measurements

FTIR of B. cereus showed the existence of O-H, C-C, CH2, C-O, C-N and C=O bands in decreasing order, Fig. 7. These bands reflect the general organic structure of bacteria which are mainly composed of polysaccharides and lipids (protein). Polysaccharides are defined from their hydroxyl bands at 3600-3200 cm-1 and carboxyl group bands at 1210-1740 cm^{-1} whereas the protein is characterized by its amino group bands at 3460 – 3150 cm^{-1} and 1650 – 1500 cm^{-1} respectively. Adsorption of B. cereus onto haematite surfaces can take place first onto their positive site of Fe^{3+} through the OH (of the polysaccharides part) and/or the COOH of both the polysaccharides or the protein fractions of the bacteria. This is confirmed from the FTIR results where a band at 3675 cm^{-1} indicated the formation of hydrogen bond after treatment of haematite with B. cereus. Such occupation of the bacteria to some of the positively adsorption sites of hematite lead to a reduction in the zeta potential of its surfaces to be close from that of the bacteria itself. The surface of hematite became therefore, more or less, hydrophobic in nature [1].

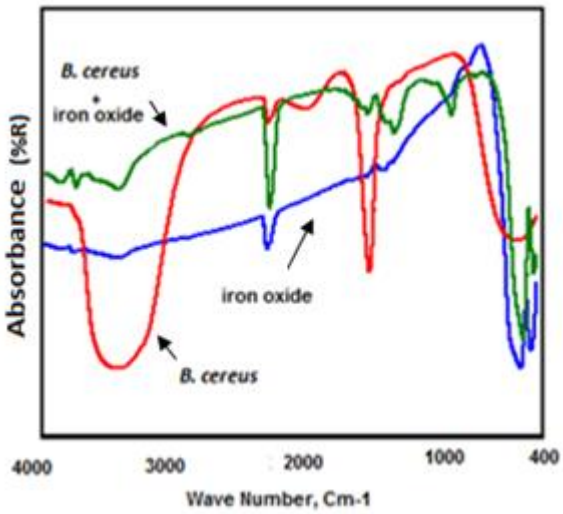

Figure 7. FTIR for B. cereus, iron oxide and its treated surface with B. cereus

HPLC and Amino acid analysis

The results of HPLC and amino acid analysis, Table 2, indicated that the total amount of saccharides secreted by *B. cereus* adsorbed onto bacteria and iron oxide surface is 83.13% ~ 507.9628 mg/g. At the same time the amino acid analysis showed that the maximum adsorption of amino acid onto iron oxide surface is 59.62%~ 3695.76 mg/gm. Actually these results are in agreement with the FTIR analysis which confirmed the hydrophobic effect of *B. cereus* onto the iron oxide surface due to the hydrogen bond formed between iron oxide surface and saccharide groups.

Table 2. Identification sheet of bacterial isolate

B. cereus (product)	Total, mg/g	*B. cereus* + Iron Oxide (mg/g)		*B. cereus* + Iron Oxide (%)	
		Residual	Adsorbed	Residual	Adsorbed
Amino acids	6196.72	2502.96	3695.76	40.38%	59.62%
Saccharides	611.024	103.0612	507.9628	16.86%	83.13%

Conclusions

- B. cereus bacteria was grown and isolated from the Egyptian iron ores collected from north Aswan locality, Egypt.
- The results showed a strong interaction between B. cereus bacteria and mineral surface of hematite. FTIR and zeta potential measurements showed a better affinity of B. cereus to hematite mineral.
- The results of zeta potential showed that the iso-electric points (IEP) for iron oxide (at pH 6.3) is significantly displaced to lower values (at pH 2.2) after it's treatment with the bacterial isolates, i.e., the IEP became very close to that of B. cereus.
- The total amount of saccharides secreted by B. cereus adsorbed onto bacteria and iron oxide surface is 83.13%. At the same time the amino acid analysis showed that the maximum adsorption of amino acid onto iron oxide surface is 59.62%. These results are in agreement with the FTIR analysis.

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