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Enhancement of Crystal Size by Microbes

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ABSTRACT

Generation of nano-crystals by microbes, both intracellularly and extracellularly, has been reported for quite sometime now. Reductases and quinones are known to facilitate this process. But little is known on the role of the microbes in enhancing the size of the crystal formed. Turbidometric method for determination of soluble sulfate concentration was used for the generation of the barium sulfate crystals both from purely chemical as well as biological (cell free supernatant) means. Transmission Electron Microscopy (TEM) was used for visualization of the stained crystal followed by measurement of the crystal size. Statistical analysis of the variation in size was done using a two-sample one-tailed Z-test. Under identical conditions of generation, the dimensions of the crystals from biological origin were much enlarged [363.54 nm (horizontal), 399.89 nm (vertical)] compared to those from purely chemical origin [265.54 nm (horizontal), 286.46 nm (vertical)]. The Z-test and computation of the corresponding p-value indicated that the variation was highly significant.

Key words: Barium sulfate, microbial enzymes, SRB, TEM, reductase, nanoparticle

INTRODUCTION

Barites whose chemical composition is Barium sulfate crystals, are commonly used for commercial applications like filler for thermosetting and thermostable plastics (to increase hardness and rigidity without disturbing the toughness and surface quality), elastomers, sealants, adhesives, varnishes, paints, paper, glass and as substrate for colored pigment formulations as well as for single-layer or multilayer coatings consisting of metal oxides, metal oxide mixtures and/or metal compounds (US Patent 7501110). They are also suitable for producing semi-opaque colourings for lamp coverings (US Patent 7501110). Medical applications include their use as X-ray contrast agent for examination of the gastrointestinal tract (US Patent 7501110; USPC 423170). It results in a distinctly higher X-ray opacity for medical articles. The particle size can be influenced by varying the concentration, temperature and stirring speed (Gardner and Nancollas, 1983).

Bacteria as well as eukarya (yeast and fungi) are known to form intracellular as well as extracellular metal micro as well as nano particles (Klaus-Joerger *et al.*, 2001; Lloyd, 2003; Kowshik *et al.*, 2003; Duran *et al.*, 2005; Nangia *et al.*, 2009; Verma *et al.*, 2010; Ahmad *et al.*, 2003). Enzymes like nitrate dependent reductase (Duran *et al.*, 2005), sulfite reductase (Kumar *et al.*, 2007; Ahmad *et al.*, 2003), nitrate reductase (Vaidyanathan *et al.*, 2010) as well as electron shuttle quinones or both (enzyme and quinines) (USPC 435168) are known to be involved in microbe based crystal generation. The Lactobacillus group of bacteria has been reported to be involved in metal nano particle generation (USPC 435168). Crystals are characterized by X-ray diffraction, transmission electron microscopy, X-ray photoelectron spectroscopy and UV-visible optical absorption (Kumar *et al.*, 2007; Haiss *et al.*, 2007; Chowdhury *et al.*, 2008; Baimark *et al.*, 2008; Lukhele *et al.*, 2010).

Soluble sulfate load in effluent water is a major industrial problem for the mining sector which calls for immediate attention. Though chemical methods are available but more emphasis has been put on the application of microbe based bioremediation (Nasipuri *et al.*, 2010). The measurement of sulfate concentration is done using turbidometric technique, (Nasipuri *et al.*, 2010; Icgen *et al.*, 2006) which results in formation of barium sulfate crystals. This study looks into the effect of the cell free supernatant from sulfate reducing bacterial consortium (isolated from East Calcutta Wetland) on the crystal size enhancement when compared to the same generated using pure chemical solutions. We ascribe the enhancement of crystal size to possible extracellular reductases.

MATERIALS AND METHODS

Sulfate reducing bacterial consortium: Media DSMZ16695 of Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH (German collection of microorganisms and cell cultures) was used for isolation of enriched SRB consortium as per the prescribed protocol (http://www.dsmz. de/microorganisms/). The molecular analysis revealed clones from genus *Desulfovibrio* among others (GenBank Accession No. GQ503854-GQ503878). The turbidometric method revealed the sulfate reducing ability (Nasipuri *et al.*, 2010). The whole study was conducted at West Bengal university of Technology.

Generation of barium sulfate crystals: The turbidometric method was used for barium sulfate crystal generation from cell free supernatant of the SRB consortium (Nasipuri *et al.*, 2010; Icgen *et al.*, 2006). The control sample was prepared by similarly treating sodium sulfate solution with one gram of barium chloride crystals. In both the cases the suspended crystals were directly used for transmission electron microscopy.

Transmission electron microscopy: Formvar coated copper grids were used for immobilization of the crystals from 20 μL of the suspension mentioned above. The grids were placed on the suspension drop for two minutes. Then the grid was placed over a drop of 0.2% uranyl acetate for 2 min. The excess stain was washed off carefully in three consecutive drops of filtered sterile milli Q water. The excess water was soaked with a blotting paper and then the grid was kept for drying overnight in a desiccator. The grids were visualized at 14000X magnification with 60 kV acceleration voltage in a Jeol JEM 100S. The images were photographed from the fluorescent screen using digital camera (Samsung S860) and printed on papers. Each crystal image was measured using vernier caliper. The measurement was carried out in both horizontal and vertical directions. The final crystal dimension was calculated taking into account the magnification of the image. Dimensions of 125 crystals were measured in both the cases.

Statistical analysis: The objective of the study was to investigate whether there was any significant difference between the dimensions (in both horizontal and vertical directions) of the crystals prepared by chemical means and of those coming from biological origin, i.e., cell free extract. In both cases (control, i.e. prepared by chemical means and sample, i.e., created from cell free extract), 125 crystals each were used for statistical analysis of the data.

The null and alternative hypotheses can be formulated as follows in case of the horizontal dimension:

$\mathbf{H_0}$: The dimension (horizontal) of the crystals originating from chemical and biological means are equal, i.e., $\mu_1 = \mu_2$

$\mathbf{H_1}$: The dimension (horizontal) of the crystals originating from biological means is greater than that originating from chemical means, i.e., $\mu_2 > \mu_1$

Sample size n = 125

α = 5%

Since, the sample size was large, a two-sample one-tailed Z-test was performed using the following formula:

$$Z = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)_{H0}}{\sqrt{s_1^2/n_1 + s_2^2/n_2}} \quad (1)$$

Where:

$\bar{x}_1$ = Sample mean of the horizontal dimensions in case of crystals from chemical origin
$\bar{x}_2$ = Sample mean of the horizontal dimensions in case of crystals from biological origin
S_1^2 = Sample variance of the horizontal dimensions in case of crystals from chemical origin
S_2^2 = Sample variance of the horizontal dimensions in case of crystals from biological origin
n_1 = Sample size for chemically prepared crystals
n_2 = Sample size for biologically prepared crystals

An exactly similar set of hypotheses was set up in case of the vertical dimension using the same sample size and level of significance.

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RESULTS AND DISCUSSION

A summary of the data obtained from measurements on the two types of crystals (Fig. 1a, b), viz., control (i.e., chemical) and sample (i.e., biological) is given in Table 1.

The result from the two-sample one-tailed test of hypothesis is shown below:

Horizontal:

$Z_{calculated}$ = -10.39 (the critical value of z at 5% level of significance is 1.64)
p-value = 0

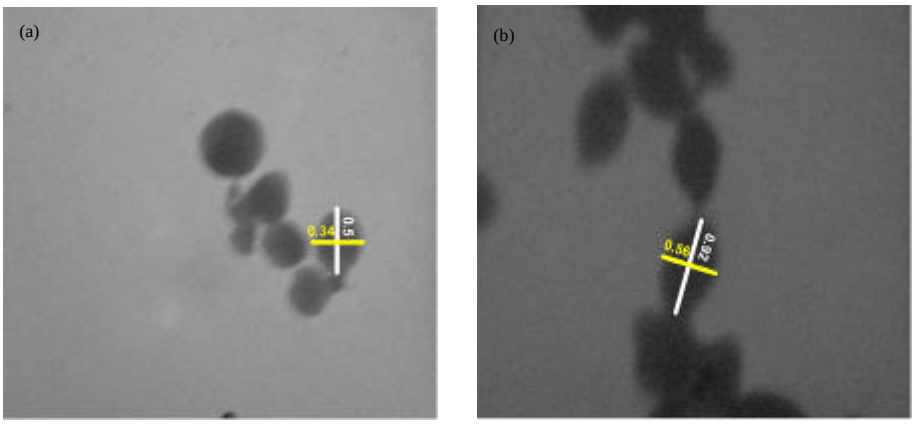

Fig. 1: Transmission electron micrograph of $BaSO_4$ crystals at 14000X magnification. $BaSO_4$ crystals were formed according to the turbidimetric method. Crystals were immobilized on formvar coated 300 mesh copper grids from Pro Sci. Tech. (GSCu300C-50) and negatively stained with 0.2% Uranyl acetate solution for 2 min. The excess stain was washed off carefully in three consecutive drops of filtered sterile milliQ water and air dried. The sample was visualized under TEM. Jeol (JEM 100S), (a) $BaSO_4$ crystals formed from Na_2SO_4 solution upon addition of 1 g of $BaCl_2$ under acidic condition according to the turbidometric method. The dimensions of the crystal are given in cm and (b) $BaSO_4$ crystals formed from cell free supernatant containing soluble sulfate according to turbidometric method. The dimensions of the crystal are given in cm

Table 1: Tabular representation of the mean and variance of the crystal dimensions generated through chemical (control) and biological (sample) means

	Crystal dimensions			
	Horizontal		Vertical	
Crystal type	Mean (cm)	Variance (cm^2)	Mean (cm)	Variance (cm^2)
Chemical	0.37	0.01	0.40	0.02
Biological	0.51	0.02	0.56	0.03

Vertical:

$Z_{calculated}$ = -8.37 (the critical value of z at 5% level of significance is 1.64)

p-value = 0

The barium sulfate crystal dimension generated in case of chemical method was 265.54 nm (horizontal) and 286.46 nm (vertical) as compared to those generated using biological means [363.54 nm (horizontal) and 399.89 nm (vertical)].

The low p-values clearly show that there is enough statistical evidence to reject the null hypotheses. This is corroborated by the fact that values of z calculated on the basis of the observed data fall in the critical region of the normal distributions. It means that the two sets (crystals obtained from chemical and biological sources) are drawn from populations which have different mean values. In other words, the dimensions (horizontal as well as vertical) of the crystals of

chemical and biological origins are significantly different from one another. This enhancement in crystal dimension could be due to the extracellular enzymes secreted by the microbial consortia. This consortium also revealed the presence of Dissimilatory Sulfite Reductase A as reported elsewhere (Nasipuri *et al*., 2010). That could be the putative reason for the enlargement phenomenon observed in case of the samples prepared by biological means keeping the other conditions identical. Here, we report for the first time the crystal dimension enhancement property of SRB consortium predominated by *Desulfovibrio* sp. Reductases from biological origin (yeast, fungus and bacteria like nitrate reducers and *Lactobacillus*) (Duran *et al*., 2005; Ahmad *et al*., 2003; Kumar *et al*., 2007; Vaidyanathan *et al*., 2010) have been reported to perform the function of crystal generation, however this study focuses on the crystal size enhancement ability of the extracellular microbial enzymes. The future extension of this study would be to fine tune the reaction to the point of fabricating barite crystals of predetermined size. An analysis of the extracellular supernatant for detection of reductase activity by biochemical means and characterization of the purified enzyme would help in understanding the process clearly.

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