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Research Article

Biogenic Synthesis of SeNPs by Probiotics Bacteria Using Modified Whey as Growth Medium and Antimicrobial Application

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Abstract

The synthesis of nano-size (~100 nm) elemental selenium by using probiotic bacterial cultures (*Lactobacillus acidophilus and Bifidobacterium*) with whey as the medium of growth revealed the usefulness of whey. Whey media was modified with whey protein concentrate (WPC 97) at 2.5%, 3.5% and 4.5% for the better growth of bifidobacteria and lactobacillus as well for augmenting the production of selenium nanoparticles. As the selenium source, Sodium Hydrogen Selenite ($NaHSeO_3$) was used at varying concentrations of 5mM, 10mM and 15mM. The colour change of whey from greenish yellow to cherry red indicated the formation of Se nanoparticle. Synthesized biogenic SeNPs were characterized by UV-vis. Spectrophotometer and TEM. Maximum absorbance of SeNPs was found at 280nm and the size was found to ~100 nm. The antimicrobial property of the synthesized selenium nanoparticle by well diffusion method against *E. coli* microorganism showed the zone of inhibition of about 21.22±0.09 mm and 21.17±0.07 mm for SeNPs produced by *Lactobacillus acidophilus and Bifidobacterium.*

Keywords: Biogenic, SeNPs, Probiotics, *Lactobacillus acidophilus*, *Bifidobacterium and* Modified Whey

INTRODUCTION

Selenium (Se), as a functional material, is an important semiconductor and photoelectric element due to its special physical properties (Zhang *et al.*, 2011). Therefore, Se is used in many applications ranging from photocells/ solar cells to semiconductor rectifiers and antioxidant to anticancer activity (Torres *et al.*, 2012; Husen and Siddiqi 2014). Se nanoparticles can be synthesized in large scale using various methods like Pulse laser ablation, electro-kinetic technique, hydrothermal treatment, vapour deposition methods. These methods required sophisticated instruments or specific chemicals which are time consuming and uneconomical chemicals or high temperature and high pressure which further pollute the environment. Normally Se is available as selenate and selenite oxoanions. The reduction of soluble Se^{4+} and Se^{6+} by microbes to insoluble non toxic elemental Se is an effective way to remove it from contaminated soil, water and drainage (Abbass and Razak 1991). Se is one of the

chalcogens occurring as selenate SeO_4^{2-}, selenite SeO_3^{2-} and selenide Se^{2-} which may be reduced to atomic state by a precursor containing an appropriate reducing agent. Biogenic synthesis of Se nanoparticles is frequently achieved by reduction of selenate/selenite in presence of bacterial proteins or plant extracts containing phenols, flavonoids amines, alcohols, proteins and aldehydes containing functional groups like ›NH, C=O, COO and C-N. Inorganic Se (selenite or selenate) also occur as selenomethionine, selenocysteine, selenocystathione, methyl selenol, dimethyl selenide and selenium methyl selenocysteine. Evidence of selenium binding protein in *Aspergillus terreus on* medium supplemented with 0.1% sodium selenite has initiated synthesis of the biogenic selenium nanoparticles using biological system (Abbass and Razak 1991).

MATERIALS AND METHOD

Materials

Whey protein concentrate - 97, was purchased from Hale and Health, puzhal, Chennai. Sodium hydrogen selenite, *Lactobacillus* MRS Agar **(**M641and M369), *Bifidobacterium* agar modified M1858 and Nutrient agar M173 were purchased from HiMedia Ltd, Bangalore. Pure *Lactobacillus acidophilus, Bifidobacterium bifidum* and *E. coli* were obtained from Central University Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai. The chemicals and reagents used were of analytical reagent grade. For all analytical purpose freshly prepared reagents were used. Whey was prepared from buffalo and cow milk obtained from the local dairy farm (Community Cattle Care Centre of CFDT, Koduvalli). Milk was heat coagulated with Acetic acid (Purchased) at different concentrations (1, 1.5 and 2%). Optimized concentration of acetic acid was used for the preparation of whey protein and concentration of Whey protein in the prepared samples were analyzed by The Kjeldahl method described by AOAC (Helrich, 1990) with standard Whey protein concentrate -97. High protein content sample was used for the preparation of Lactobacillus acidophilus and Bifidobacteria agar medium.

Biogenic synthesis of Selenium nanoparticles

Lactobacillus acidophilus and *Bifidobacteria* agar and broth were prepared based on earlier described method with slight modifications (Eszenyi et al., 2011). Prepared mediums were sterilized by autoclaving for 15 psi (121^{o}C) for 15 min, cooled to 50^{o}C for the preparation of agar plate. *Lactobacillus acidophilus* was cultured on MRS broth and agar respectively by incubating at temperature of 37^o C/24-36 hrs. The *Bifidobacterium bifidum* was cultured in bifido broth and bifido agar (Suresh et al., 2010) under anaerobic conditions using Anearo gas pack (Hi media LE002A-5NO) at a temperature of 39 - 42^o C /24-72 hrs for the isolation of bifidobacterial cultures. Similarly, 5, 10 and 15mM as final concentration of sodium hydrogen selenite ($NaHSeO_3$) was prepared with both MRS and bifido broth and 1 ml of each culture was added separately for the synthesis of selenium nanoparticles under the room temperature. The color changes were continuously monitor and measured. The synthesized selenium nanoparticles characterized by UV-visible spectrophotometer and TEM. FIG 3

Antimicrobial assay

The antimicrobial activity of produced nanoselenium was screened using agar well diffusion technique in nutrient agar was used for the antimicrobial studies. The agar plates were prepared by pouring 15 ml of molten agar media into sterile petriplates. The plates were allowed to solidify and 0.1 % inoculums of *E.coli* were swabbed uniformly with sterile cotton and were allowed to stand for 15 minutes. The produced nanoselenium of 1µl was loaded in the well. The surface was allowed to dry for 5 minutes and the plates were incubated at 37°C for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with ruler in millimeter. These studies were performed in triplicate.

RESULTS AND DISCUSSIONS

Assessing the quality of whey samples

Whey, normally obtained as a by-product of dairy industry is wasted and is a threat to environment as a pollutant. Its usefulness as a growth media for elemental nano selenium production will be a boon to the dairy industry. Whey samples were prepared by heat coagulation of mixture of buffalo or cow milk with acetic acid at different concentration levels (1, 1.5 and 2%) with pH ranged from 6.4 to 6.7. The clarity of whey obtained from buffalo milk at 2% of acetic acid treatment shows better clarity while comparing the other concentrations as discussed in Table 1. Subsequent addition of whey protein concentrate to whey under sterile environment helps for culturing by *L.acidophilus, B.bifidum.* The clarity of whey after autoclaving and its pH were considered for initial screening to use as a growth media in the process of production of elemental nano selenium.

	Type of whey	**pH**	**Clarity of whey after autoclaving**
BWa_1	Buffalo milk whey AA $_1$	6.5	Slightly opaque
$BWa_{1.5}$	Buffalo milk whey AA $_{1.5}$	6.5	Transparent
BWa_2	**Buffalo milk whey AA $_2$**	**6.7**	**Better clarity and transparent**
CWa_1	Cow Milk whey AA $_1$	6.5	Slight Precipitate
$CWa_{1.5}$	Cow Milk whey AA $_{1.5}$	6.5	Slightly opaque
CWa_2	**Cow Milk whey AA $_2$**	**6.5**	**Clear and transparent**

Table 1: Assessing the quality of whey samples

Sl.no	**Sample**	**WPC 97 in gm per 100 ml**	**Protein content**	**F value**
1	**P1**	**2.5**	**3.01 ^{c} ±.00**	27.442**
	MCW	2.5	2.93 ^{a} ±.01	
	MMW	2.5	2.97 ^{b} ±.01	
2	**P2**	**3.5**	**3.99 ^{c} ±.00**	89.115**
	MCW	3.5	3.91 ^{a} ±.00	
	MMW	3.5	3.96 ^{b} ±.01	
3	**P**	**4.5**	**5.02 ^{d} ±.02**	82.145**
	MCW	4.5	4.80 ^{a} ±.00	
	MMW	4.5	4.94 ^{b} ±.01	
4	Buffalo milk whey	Control	0.29 ^{c} ±.01	22.226 **
	Cow milk whey	Control	0.19 ^{a} ±.01	
	Mixed milk whey	Control	0.23 ^{b} ±.01	

Table 2: Protein content of modified whey samples

Probiotic Culture	**Time interval (Hrs)**						
	0	6	12	18	24	30	36
La	-	-	+	+	++	++	+++
Bb	-	-	+	++	++	+++	+++

\+ *Light change,* ++ *Medium red change,* +++ *Cherry red colour*

Table 3: Colour intensity development at various incubation periods

Concentration of $NaHSeO_3$	*L.acidophilus cultured sample*	Colour intensity	*B.bifidum cultured sample*	Colour intensity
5mM	S_1	++	S_1	++
10mM	S_2	++	S_2	++
15mM	S_3	+++	S_3	+++

\+ *Light change,* ++ *Medium red change,* +++ *Cherry red colour*

Table 4: Colour intensity development at various levels of addition of $NaHSeO_3$

Protein content of modified whey samples

WPC at varying levels (2.5, 3.5and 4.5) were added to 100ml of buffalo milk, cow milk and mixed milk whey to get the modified whey of P, MCW and MMW. And samples were taken for protein estimation which reveals higher significant difference as shown in Table 2. Among these, buffalo milk shows higher protein content than the modified cow milk (MCW) and mixed milks MMW with significant difference. High protein content of 5.02 per cent was used for further study. Capela *et al.* (2006) opined that the addition of substances such as whey protein in dairy products enhanced the viability of probiotics probably due to their buffering property. Therefore, the WPC were added to modify the whey as the culture media for the growth of probiotic cultures for the production of nanoselenium.

Biogenic synthesis of SeNPs and characterizations

Greenish yellow color of solution was changed to red color after 12hrs incubation of *Lactobacillus acidophillus and Bifidobacterium bifidum* with different concentrations of sodium hydrogen selenite. The color changes indicate the formation of selenium nanoparticles. The intensity red color of biogenic selenium nanoparticles was increased while increasing incubation time up to 36 hrs as summarized in Table 3. This also leads to increase the biomass of *Lactobacillus acidophillus and Bifidobacterium bifidum.* This increased biomass increases the rate of reduction of selenium (Se^{+4}) to selenium nanoparticles (Se^0) with help of NADH – dependent cytosolic/intracellular and membrane bound oxidoreductase enzymes (Daniela *et al.*, 2014). At optimized time of incubation, biogeneic synthesis of selenium nanoparticles was increased by increasing the concentration of sodium hydrogen selenite from 5, 10, 15 mM, respectively (Table 4). This is due to the reduction of more selenium ions in to selenium nanoparticles and the intensity of the colour is proportionate to the concentration of nano selenium produced by *L.acidophilus* and *B.bifidum* (Eszenyi *et al.* 2011; Zahra *et al.* 2010; Keka *et al.* 2011). In addition, pH changes in the culture medium are not affecting the synthesis of SeNPs. Figure 1 shows the cherry red color of synthesized SeNPs in different concentration of selenium salt (S1- 5 mM, S2- 10 mM, and S3- 15 mM) by *L.acidophilus* and *B.bifidum.* UV- visible absorption spectra of synthesized SeNPs as shown in Figure 2 A and B.

Absorbance maximum of biogenic SeNPs synthesized by *L.acidophilus* was found at 280 to 281 nm. The intensity of absorbance peak was increased to 0.6, 0.8 and 0.9 with increase the concentration of sodium selenite from 5mM, 10 mM and 15 mM in the presence of probiotics *L.acidophilus* as shown in Figure 2A UV-visible spectra a-c, respectively. Spectra'd' is the control without probiotic culture show low absorbance (0.273) at 281 nm. This confirms that the production of biogenic SeNPs by *L.acidophilus.* Figure 2B, shows similarly optical absorbance properties of SeNPs produced by *B.bifidum.* The intensity of peak for 5mM, 10mM and 15 mM was found to be 0.204, 1.153 and 1.003 in spectra 'a-c', respectively. These peaks intensity were found to be higher than the spectra's' evidenced that the presence of SeNPs produced by probiotics bacteria's through the reduction of selenium ions to elemental selenium. Figure 2C bar graph reveal that the potential of SeNPs synthesis by

L.acidophilus and *B.bifidum.* These finding has good argument with previous report (Praharaj *et al.,* 2006).

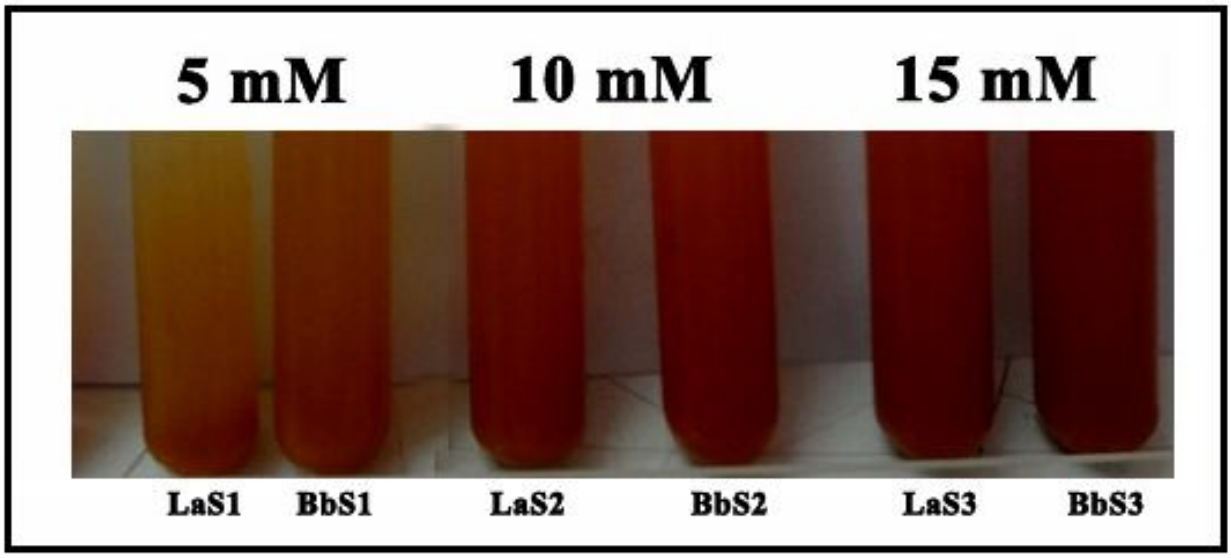

Figure1: Nano Selenium production by different cultures at different levels of sodium hydrogen selenite

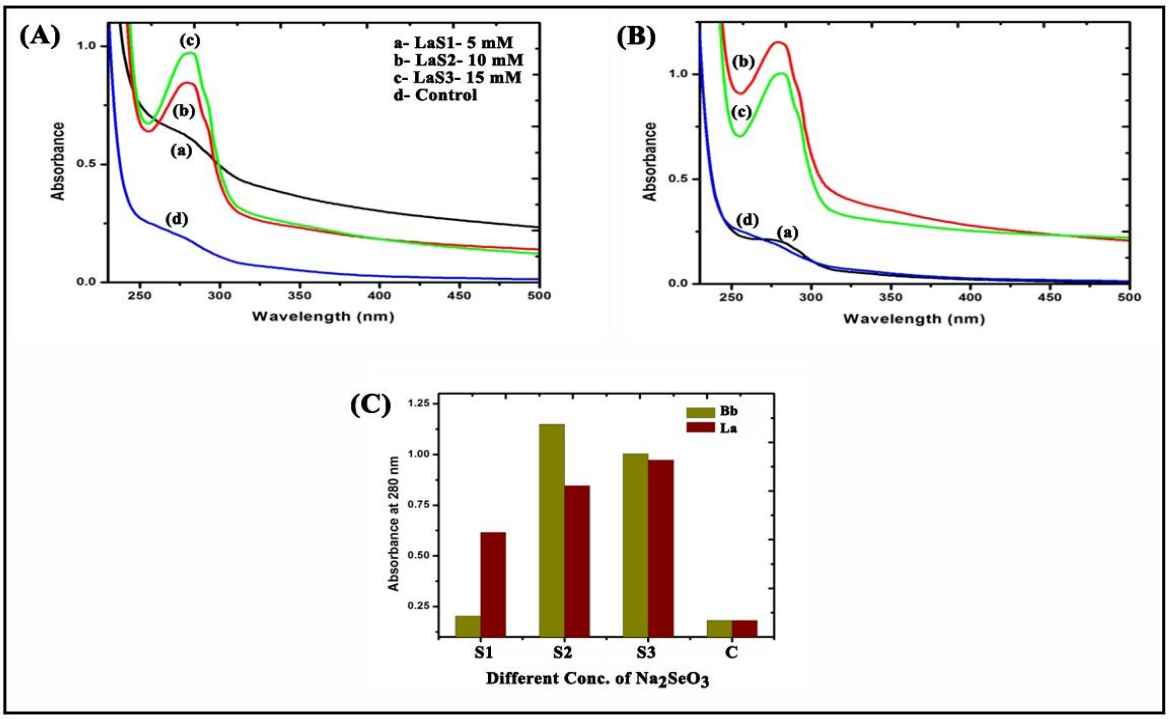

Figure 2: UV- visible absorption spectra of synthesized SeNPs

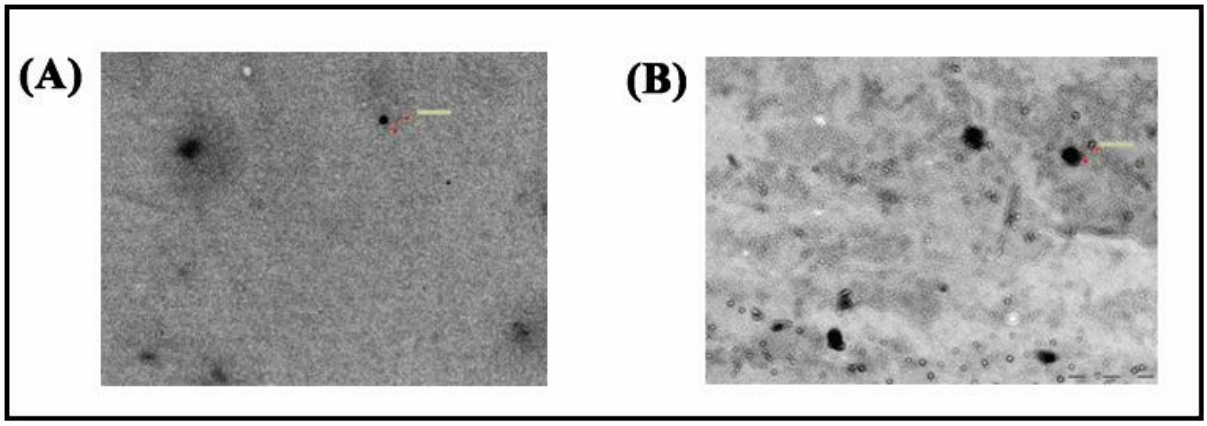

Figure 3: TEM characterization of SeNPs nanosphere

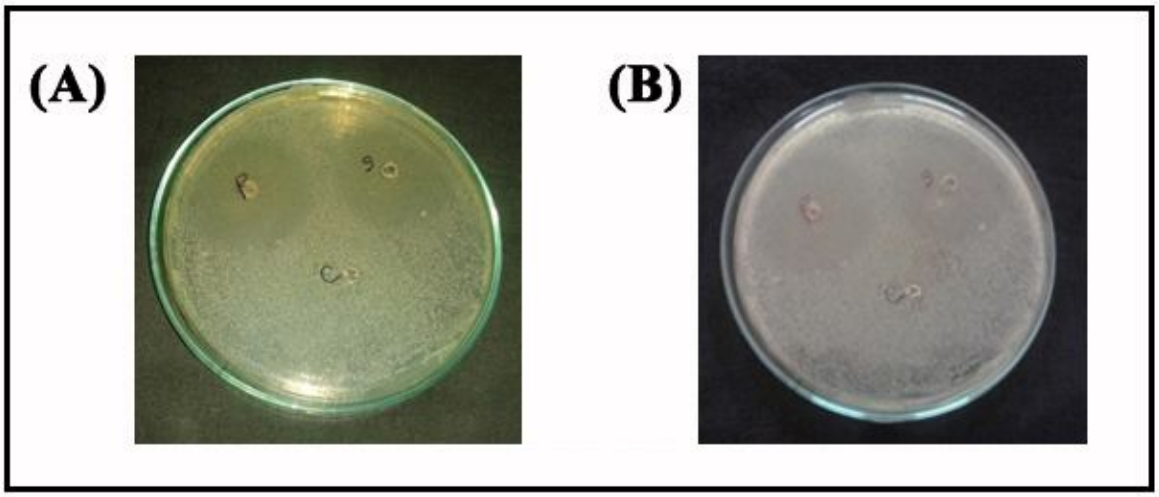

Figure 4: Zone of inhibition of *E. coli* growth was found to be formation 21.22± .09mm for SeNPs produced by *L. acidophilus* and 21.17 ±.07 mm for SeNPs produced by *B. bifidum*

The size and shape of synthesized SeNPs was characterized by TEM. Figure 3Aand B showed the dispersed SeNPs nanosphere with spherical shaped produced by *L. acidophilus* and *B. bifidum*. The size of SeNPs was found to 115.004 nm and 132.74 nm, respectively. This is due to the nucleus growth of reduced elemental selenium and capped by bacterial proteins in defined shape and size. This is proved that probiotic bacteria reduced the selenium ions to metal and capped by its proteins (Soniya *et al.,* 2010).

Antimicrobial property against *E. coli*

Antibacterial activity of synthesized SeNPs was investigated using *E.coli* through well diffusion method (Table 5). Supernatants of centrifuged SeNPs has used for this study. Zone of inhibition of *E.coli* growth was found to be formation 21.22± .09mm for SeNPs produced by *L.*

acidophilus and 21.17 ±.07 mm for SeNPs produced by *B.bifidum* (Figure 3A and B). This is due to the intracellular accumulation of SeNPs and its oxidized selenium oxide in *E.coli* which could be interfere with normal cellular function leads to cell death (Erfan et al.2014).

Samples	Zone diameter
Lap s3	21.22± .09
Bbp s3	21.17 ±.07
T-Test	0.432 ns

Table 5: Antimicrobial property against *E. coli*

CONCLUSION

Whey, normally obtained as a by-product of dairy industry is wasted and is a threat to environment as a pollutant. Its usefulness as a growth media for elemental nano selenium production will be a boon to the dairy industry. Spherical shaped nano sized SeNPs was synthesized using *Lactobacillus acidophilus* and *Bifidobacterium* with modified whey as growth medium. UV-vis spectrophotometer and TEM analysis evidenced that the presence of SeNPs in the growth medium produced by probiotic bacteria's through the reduction of selenium ions to elemental selenium with aid of intracellular reductase enzymes. Potential of SeNPs synthesis by *Lactobacillus acidophilus* was slightly greater than *Bifidobacterium.* Also, *L. acidophilus* produced SeNPs showed strong antimicrobial property than the *Bifidobacterium* produced SeNPs against *E. coli*. This study will improve the utility of diary waste whey protein for the cultivation of probiotics and production of SeNPs for antimicrobial application against various microorganisms.

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