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ECO-FRIENDLY SYNTHESIS OF SILVER NANOPARTICLES USING *Lactobacillus delbrueckii* subsp. *bulgaricus* ISOLATED FROM KINDRIMO (LOCALLY FERMENTED MILK) IN KANO STATE, NIGERIA

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ABSTRACT
Silver nanoparticles have gained global popularity because of its application in crucial areas such as medical diagnosis/therapy, solar cell development, water treatment, surface coating and cosmetic production. Bacterial synthesis of silver nanoparticles is regarded as eco-friendly due to minimal waste generated while being energy efficient. This study was aimed at synthesizing silver nanoparticles using Lactobacillus specie isolated from Kindrimo under the influence of sunlight irradiation. Lactobacillus delbrueckii subsp. bulgaricus isolated from Kindrimo was identified using morphological and biochemical techniques and confirmed using 16S rRNA gene sequencing. The eco-friendly protocol for the extracellular synthesis of silver nanoparticles was accomplished by treatment of Lactobacillus delbrueckii subsp. bulgaricus extract with silver nitrate solution in the ratio 1:10 under the influence of sunlight irradiation. Synthesized silver nanoparticles were confirmed and characterized using UV-Visible Spectrophotometry, Dynamic Light Scattering, Transmission Electron Microscopy and Energy Dispersion Spectroscopy. The absorbance spectrum preliminarily confirmed the formation of silver nanoparticles by revealing characteristic broad peak at 415nm. Dynamic Light Scattering measurements further confirmed synthesis by showing average particle size distribution of 147nm. Micrograph of Transmission Electron Microscopy revealed that the synthesized silver nanoparticles were polyhedral and spherical in shape with size ranging 1.4 to 8.9nm. The elemental composition of the resultant colloidal solution was shown to contain silver nanoparticles when subjected to Energy Dispersion Spectroscopy. This study proved that the effect of sunlight irradiation on Lactobacillus mediated synthesis of silver nanoparticles is energy efficient and inexpensive.
Keywords: Silver nanoparticles; UV-Visible Spectrophotometry; Dynamic Light Scattering; Transmission Electron Microscopy; Energy Dispersion Spectroscopy

INTRODUCTION

Nanotechnology has emerged as multidisciplinary discipline with physics, chemistry, biology, material science, medicine and engineering playing active roles (Chaudhari *et al.*, 2012). Development of nanomaterial is a major facet of Nanotechnology. Nanomaterials exhibit unique physical/chemical properties and impart enhancements to engineered materials, including enhanced magnetic, electrical activity, and optical properties. These outstanding attributes have resulted in worldwide increase in application and investment in nanomaterial research.

Although several numerous innovative protocols and characterization techniques exist for nanomaterial synthesis, there is currently a drive forthe development of eco-friendly technologies that accomplishes synthesis within a short time frame, consumes minimal energy, produces minimal waste with less toxic effect (Sarvamangala *et al.*, 2013). Bacterial synthesis of nanoparticles is regarded as eco-friendly because the processes involved generate minimal waste with subdued toxic consequence to the environment. This is in addition to being commercially economical. It is reported that bacterial synthesis is advantageous over other types of synthesis because the biological bases allows nanoparticles to be biocompatible with living systems thereby reducing risk when used for medical purposes.

The principle behind the successful use of bacteria in the synthesis of nanoparticles lies in the process of bio-reduction in which metal ions present in metal salts accept electrons from NADH dependent bacterial enzymes thereafter resulting in the formation of stable and inert metallic nanoparticles (Pantidos and Horsfall 2014).

The unique optical, electrical, conductive, thermal and antibacterial properties of silver nanoparticles have made them suitable for many industrial applications as such it is being rated as being amongst the most commercialized metallic nanoparticles. Quite a number of studies have reported either the extracellular or intracellular synthesis of silver nanoparticles using *Lactobacillus* specie isolated from various sources (Dhoondia and Chakraborty, 2012; Chaudhari *et al.,* 2012, Senthil Prabhu *et al.,* 2014; Ranganath *et al*., 2012). Despite this feat, the protocol suffers from the drawback of high energy cost (temperature) and long synthesis period with some studies reporting time frame of 3 - 5 days for the synthesis of silver nanoparticles. This major shortfall triggered further studies on possible ways to reduce the reaction time lag for rapid synthesis of nanoparticles. The use of sunlight irradiation has been explored as a means to enhance the rate of biological synthesis of silver nanoparticles. Brahmachari *et al*. (2014) reported that the rapid synthesis of silver nanoparticles that explored sunlight irradiation in addition to biological synthesis was a simple and efficient one-step protocol.

In this paper, *Lactobacillus delbrueckii* subsp. *bulgaricus* was isolated from Kindrimo (local fermented milk in Kano State of Nigeria) and was exploited for rapid and eco-friendly synthesis of silver nanoparticles under the additional influence of sunlight irradiation. The eco-friendly protocol developed led to the synthesis of silver nanoparticles in 10 minutes.

MATERIALS AND METHODS

Isolation and Identification of *Lactobacillus delbrueckii* subsp. *bulgaricus* from Kindrimo

Samples of Kindrimo were obtained from settlements of Fulani nomadic herdsmen of Kano State, Nigeria. For each sample, 1ml of Kindrimo was mixed with 9ml of distilled water, homogenized and serial diluted. One ml of 10^{-4} was pour plated aseptically on MRS agar and subsequently incubated at 37°C for 48 hours in anaerobic condition using anaerobic jar. After the incubation, distinct colonies were streaked on the fresh MRS agar petri plate and subsequently incubated at 37°C for 48 hours for the formation of isolated colonies. The morphological characterization was performed according to Bergey's Manual of Determinative Bacteriology as such Grams staining, endospore staining and catalase test were preliminarily used for the identification. API 50 CHL System (API CH strip and API 50 CHL Medium) manufactured by Biomerieux SA, France was used for the biochemical identification. Molecular identification was carried out via 16S rRNA gene sequencing to further substantiate result. Bacteria that was identified as *Lactobacillus delbrueckii* subsp. *bulgaricus* was purified and preserved on MRS agar slants and stored at 4°C for further analysis.

*Lactobacillus delbrueckii*subsp. *bulgaricus* Mediated Synthesis of Silver Nanoparticles

The study was carried out at Federal University of Technology, Bosso Campus, Minna in Niger State of Nigeria (latitude 9 ° 41' N and longitude 6 ° 31' E; 258.5 m above sea level) during the months May and June with mean temperature of about 33.5°C (Afolabi *et al*. 2014).A loopful of pure colonies of *Lactobacillus delbrueckii*subsp. *bulgaricus* were picked and inoculated into nutrient broth and incubated at 37°C for 24 - 48 hours. After incubation, developed biomass was placed in a shaker at 25°C for 30 minutes and subsequently centrifuged at 5,000 rpm for 10 minutes. The supernatant was separated and collected in sterile reagent bottles and stored at 4°C for further use in silver nanoparticle synthesis (Chaudhari *et al.,* 2012).

In a procedure described by Senthil-Prabhu*et al*. (2014), 1ml of supernatant from *Lactobacillus delbrueckii* subsp. *bulgaricus* was mixed with 10ml of 1mM of $AgNO_3$. The reaction mixture was stirred properly and exposed to direct sunlight on a bright sunny day for 10 minutes (Das *et al*., 2016). Ten Milliliter (10ml) of $AgNO_3$ and 10ml of supernatant separately were used as control as such were placed alongside the reaction mixture. Within seconds of exposure to sunlight the reaction mixture turned from whitish to reddish brown indicating presumptive formation of silver nanoparticles. Furtherance to this, the reaction mixture was incubated for 24 hours at room temperature to allow for stabilization.

Characterization of Synthesized Silver Nanoparticles

Further confirmation of synthesized silver nanoparticles and characterization entailed the use of four bioinstrumentation techniques. UV-Visible Spectrophotometry and Dynamic Light Scattering were carried out at the Centre for Genetic Engineering and Biotechnology, Federal University of Technology, Bosso Campus, Minna in Niger State of Nigeria. Transmission Electron Microscopy (TEM) and Energy Dispersion Spectroscopy (EDS) was carried out at the Electron Microscope Unit, Department of Physics, University of the Western Cape, South Africa. UV – Visible Spectrophotometry is a proven technique used for the detection of silver nanoparticles and the reduction of silver ions was detected by measuring the absorbance spectra of 2ml of aliquot of the reaction mixture after 24 hours by scanning between 190nm and 600nm using UV – 1800 UV Spectrophotometer by Shimadzu Corporation, Japan. Adopting Barkat*et al*. (2014) and Ishaq *et al*. (2015), Dynamic Light Scattering (DLS) which is a technique that utilizes laser diffraction with multiple scattering was used to determine the particle size distribution of the synthesized silver nanoparticles in the reaction mixture by employing the use of a Zetasizer Nano-S Instrument (Malvern Instruments, Nano S) operating with a 532nm laser. TEM was used to confirm the synthesis of silver nanoparticles as per standard procedures with the purpose of obtaining measures of particle size and morphology. Sample for TEM analysis were prepared on a carbon coated grid by dropping a minute amount of the sample on the grid. Extra solution was removed using blotting paper and then, the film on the TEM grid was allowed to dry by putting it under a mercury lamp for 5 minutes (Omidi*et al*., 2014).Authentication of the formation of elemental silver by *Lactobacillus delbrueckii*subsp. *bulgaricus* was finally confirmed through EDS. This was realized with the use of a field emission gun attachment equipped with the Transmission Electron Microscope.

RESULTS AND DISCUSSION

Isolation and Identification of Bacterial Isolate from Kindrimo

Kindrimo served as the source for the isolation of *Lactobacillus* in this study. Kindrimo was cultured on MRS agar and the bacteria isolated was revealed to be Gram positive, endospore negative and catalase negative as presented in Table 1. Reference was made to criteria set in Bergey's Manual of Determinative Bacteriology and the genus of isolated bacteria was characterized as *Lactobacillus*. *Lactobacillus* species are present in raw milk and dairy products such as cheeses, yoghurts and fermented milk (Ranagath *et al*., 2012). Kindrimo is described by Igwe *et al*. (2014) as a viscous full fat (or partially skimmed) traditionally fermented milk native to the nomadic herdsmen of the Fulani tribe of Nigeria. Kindrimo as local yoghurt in Nigeria was therefore termed a good source for the isolation of *Lactobacillus* specie.

Table 1: Morphological Identification of *Lactobacillus*

Morphological Identification	Result
Colour	Creamy-white
Colony formation	Smooth elevated colonies
Shape	Thick long bacilli
Gram Reaction	+
Endospore Test	-
Catalase Test	-

The use of API 50 CHL system for biochemical characterization through analysis of fermentation pattern of 49 sugars inferred that the isolate was *Lactobacillus delbrueckii* subsp. *bulgaricus*. This is depicted in Table 2. The result of the molecular characterization using 16S rDNA gene sequencing further authenticated the bacteria. Figure 1 presents the partial gene sequence of the isolated bacteria which showed 99% sequence homology with *Lactobacillus delbrueckii* subsp. *bulgaricus*. In recent years, identification of microorganisms has moved from phenotypic to genotypic methods as they yield more sensitive and accurate results.

Table 2: Biochemical Identification of *Lactobacillus*

Sugar Source	N1
Glycerol	-
Erythriol	-
D-arabinose	-
L-arabinose	-
Ribose	-
D-xylose	-
L-xylose	-
Adonitol	-
β-metil-D-xyloside	-
Galactose	+
D-glucose	+
D-fructose	+
D-mannose	-
L-sorbose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Mannitol	-
Sorbitol	-
α-methyl-D-mannoside	-
α-methyl-D-glucoside	-
N-acetyl-glucosamide	-
Amigdalin	-
Arbutin	-
Esculin	-
Salicin	-
Cellobiose	-
Maltose	-
Lactose	+
Melibiose	+
Saccharose	-
Trehalose	-
Inulin	-
Melezitose	-
D-raffinose	-
Amidon	-
Glycogen	-
Xylitol	-
β-gentiobiose	-
D-turanose	-
D-lyxose	-
D-tagarose	-
D-fuccose	-
L-fuccose	-
D-arabitol	-
L-arabitol	-
Gluconate	-
2-keto-gluconate	-
5-keto-gluconate	-
Inference	*Lactobacillus delbrueckii subsp. bulgaricus*

99% IDENTICAL to *Lactobacillus delbrueckii subsp. bulgaricus* strain MGD9-4
16S ribosomal RNA gene, partial sequence

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AAACGCTGGGGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAATTCAAAGATCCCTTCGGGGTGATTGTTGGACG
CTAGCGGCGGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGGATACCACTTGGAAACAGGTGCTAAT
ACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGTAAGCTGTCACTTTAGGATGAGCCCGCGGCG
CATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCAATGATGCGTAGCCGAGTTGAGAGACTGATCGGCCACATTG
GGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGCAGTAACTGGT
CTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCACGGCTCAACCGT
GGAACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGGAATGCGTAG
ATATTGGAAGAACACCAGTGGCGAAGGCGGCTCCCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGA
CAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGCGCTAGGTGTTGGGGACTTTCCGGTCCTCAGTGCCG
CAGCAAACGCATTAAGCGCTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTGCGCTACACCTA
GAGATAGGTGGTTCCCTTCGGGGACGCAGAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTCTTTAGTTGCCATCATTAAGTTGGGCACTCTAAAGAGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGC
AGTACAACGAGAAGCGAACCCGCGAGGGTAAGCGGATCTCTTAAAGCTGTTCTCAGTTCGGACTGCAGGCTGCAACT
CGCCTGCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGAAGTCTGCAATGCCCAAAGTCGGTGGGATAACCTTTATAGGAGTCAGCCGCCTAAGGCA
GGGCAGATGACTGGGGGGACT
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Fig. 1: 16S rRNA partial sequence indicating *Lactobacillus delbrueckii* subsp. *bulgaricus*

Synthesis and Characterization of Silver Nanoparticles using *Lactobacillus delbrueckii* subsp. *bulgaricus*

The ability of *Lactobacillus delbrueckii* subsp. *Bulgaricus* to synthesize silver nanoparticles was achieved by a reaction between extract of *Lactobacillus delbrueckii* subsp. *Bulgaricus* and 1mM $AgNO_3$ in the ratio 1:10 and exposure to sunlight. Plate 1 shows the colour change of the reaction mixture from white to reddish brown after exposure to sunlight for 10 minutes. Both controls ($AgNO_3$ only and supernatant only) which was placed alongside remained unchanged. The peculiarity of this study was the utilization of sunlight radiation to synthesize silver nanoparticles using *Lactobacillus* as a reducing agent. A number of studies exist which reports the synthesis of silver nanoparticles by employing a reducing agent (majorly plant extract) and sunlight. Das *et al*. (2016) reported employing sunlight irradiation induced strategy for the rapid synthesis of silver nanoparticles which were in the range of 70 -90 nm with spherical shape whilst using glycolipid bio-surfactant extracted from *Pseudomonas* sp. as a reducing agent.

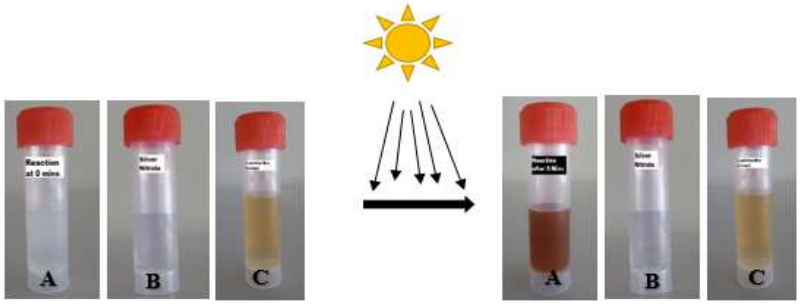

Plate 1: Before and after *Lactobacillus* mediated synthesis of silver nanoparticles.
A: Reaction mixture of *Lactobacillus* + $AgNO_3$, B: $AgNO_3$ (Control), C: *Lactobacillus* supernatant (Control).

On subjection of the developed biomass of *Lactobacillus delbrueckii* subsp. *bulgaricus* mediated synthesis of silver nanoparticles to UV-Visible spectrophotometry, wavelength peaked broadly at 415nm which is characteristic peak for silver nanoparticles. Figure 2 presents the result of the UV-Visible spectra analysis which shows remarkable difference between the reaction mixture and both controls. The results obtained are in accordance with studies such as Jaffat *et al*. (2017)in which *Lactobacillus* specie was successfully used to synthesize silver nanoparticles which exhibited maximum absorbance at 410nm in UV-Visible spectrophotometry. This characteristic absorption peak is reported to be an indication of the formation of silver nanoparticles. Several authors have reported peaks of absorption spectra of silver nanoparticles in the range 391–440 nm, while the peak at 292 nm could be attributed to the presence of proteins (Lateef *et al.*, 2015).

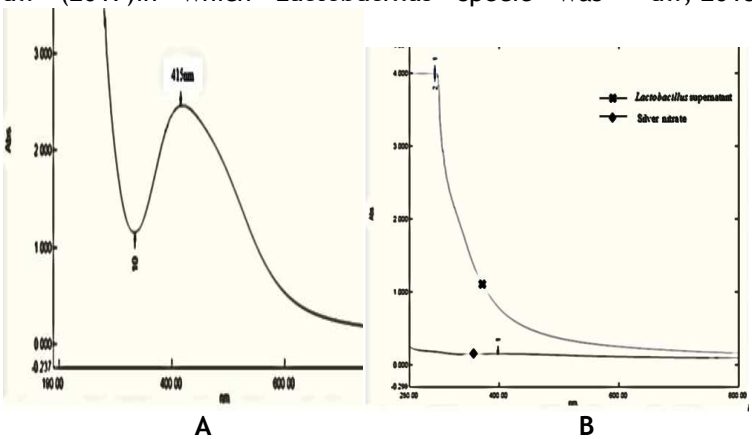

Fig.2: A=UV Spectra of biomass containing synthesized silver nanoparticles, B=UV Spectra of Control (*Lactobacillus* supernatant and silver nitrate)

The reaction mixture containing formed silver nanoparticles were subjected to Dynamic Light Scattering (DLS) measurements as shown in Figure 3and it revealed that the average particle size *Lactobacillus delbrueckii* subsp. *bulgaricus* was 147nm and monodispersed. Dynamic light scattering which employs the use of a particle size analyzer provides extra detail about the particle nature, such as monodispersed, didispersed and polydispersed whilst determining the sizes and distribution of nanoparticles formed. This result shares similarity with Natarajan *et al.,* (2010) in with it was reported that the use of Laser diffraction particle size analyzer to characterize silver nanoparticles produced by *E. coli* revealed various sizes range from 40 to 60 nanometers in a polydispersed mixture.

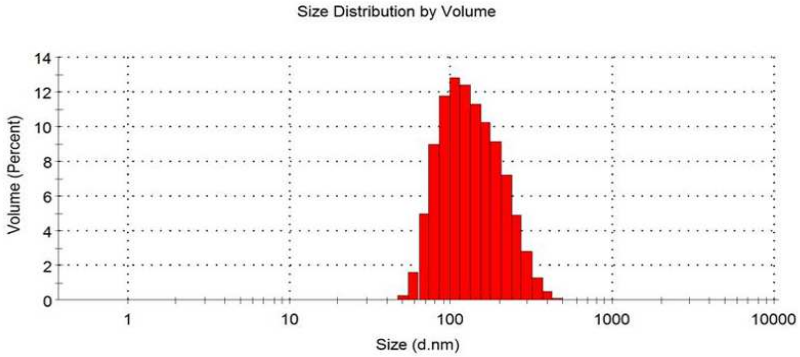

Fig. 3: Particle size distribution of silver nanoparticles synthesized by *L. delbrueckii*subsp. *bulgaricus*

TEM was used to determine the morphology (in terms of size and shape) of silver nanoparticles synthesized by *Lactobacillus* isolated from Kindrimo. The micrograph is presented in Plate 2 and it revealed the variability in the morphology of nanoparticles synthesized. Spherical forms were dominant with few polyhedral forms present. The particle size ranged from 1.4 to 8.9nm. The analysis is consistent with Dhoondia and Chakarborty (2012) in which the TEM images of the silver nanoparticles synthesized by *Lactobacillus* showed some variability in shape and size which range between 2 -20nm. TEM has been used in several studies to ascertain the size and shape of silver nanoparticles (Omidi *et al.,* 2014).

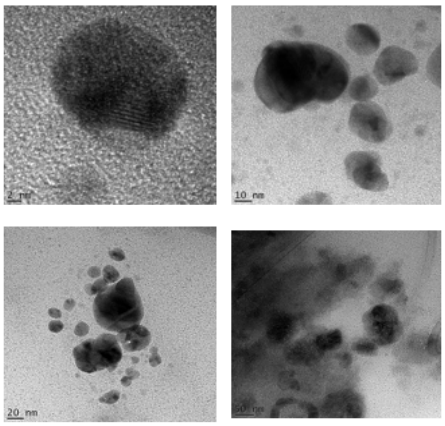

Plate 2: TEM micrograph of silver nanoparticles synthesized by *L. delbrueckii subsp. bulgaricus*

EDS was used to analyze elemental composition of the reaction mixture after bioreduction and it revealed silver nanoparticles in significant amount as shown in Figure 4. The other elements detected may have emanated from either the media used to culture the bacteria or the Polyethylene Glycol that was used to process the reaction mixture for characterization. An analysis of EDS spectrum can provide quantitative and qualitative status of the elemental composition of a sample. Silver nanoparticles usually exhibit optical absorption signals at 3keV, which could be due to the surface plasmon resonance (SPR) (Ranganath *et al.*, 2012).

In research by Sarvamangala *et al*. (2013), EDS analysis proffered evidence for the reduction of silver ions to silver nanoparticles by *Lactobacillus bulgaricus*. Chaudhari *et al*. (2012) and Senthil-Prabhu *et al*. (2014) also utilized EDS to positively confirm silver nanoparticles produced by *Lactobacillus* species.

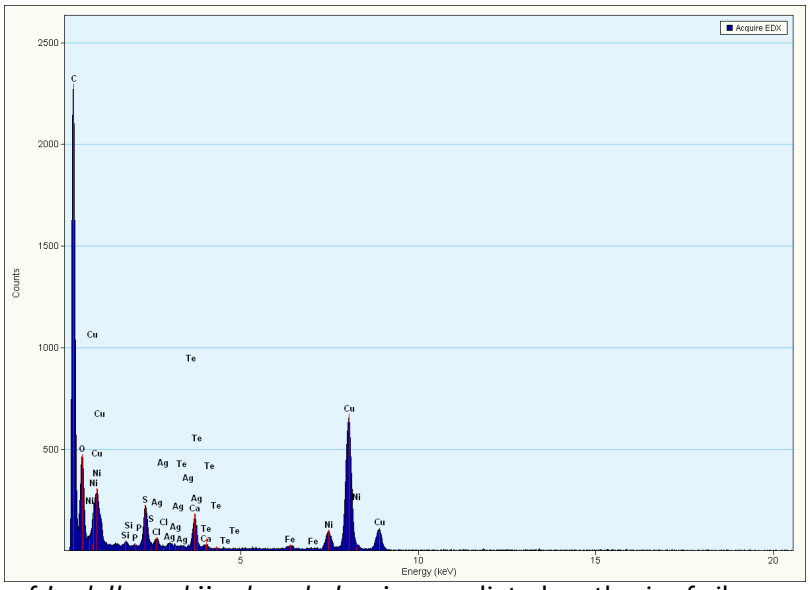

Fig. 4:EDS analysis of *L. delbrueckii subsp. bulgaricus* mediated synthesis of silver nanoparticles

CONCLUSION

This study developed an eco-friendly protocol for the synthesis of silver nanoparticles using extract from *Lactobacillus delbrueckii* subsp. *Bulgaricus* under the influence of sunlight irradiation.The size of silver nanoparticles synthesized ranged from 1.4 to 8.9nm and the shape was predominantly spherical. The synthesis was rapid and minimal waste was generated.

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