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Bacterial diversity study for synthesis of copper nanoparticles

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Abstract
Biological synthesis of nanoparticles has come out as fastly growing area of research in nanotechnology across the globe. This method is an alternate to the conventional methods such as physical and chemical approach. Microorganism and plant parts are used for the fabrication of nanoparticles but bacterial synthesis is gaining more importance over the plant and fungal synthesis of nanoparticles. In present study, samples were collected from the copper rich sites of Himachal Pradesh and Rajasthan for studying the bacterial diversity for synthesis of copper nanoparticles. Total 154 bacterial isolates were isolated using nutrient agar medium and characterized by using morphological and biochemical characters. All isolates were quantitatively screened by using 2mm copper sulphate and ability of synthesizing copper nanoparticles were primarily confirmed by colour change from light blue to greyish blue.

Keywords: Biological, nanoparticles, copper, synthesis and nanotechnology

Introduction
Various metal nanoparticles were synthesized by physical and chemical methods but both methods have disadvantages such as it is costly process, produce toxic by-product etc. Disadvantages of both physical and chemical methods for fabrication of nanoparticles greatly limit their applications in various fields and therefore development of reliable, nontoxic and eco-friendly technologies for fabrication of nanoparticles are of utmost importance to expand their application. To achieve the goal of synthesis of nanoparticles microbial synthesis is the one of the alternative methods, which leads to another new branch of nanotechnology and is "bio-nanotechnology" which is defined as understanding and control of the matter at dimensions of roughly 1-100 nm, where properties of matter differ fundamentally from those of individual atoms or molecules or bulk material. The use of microorganisms to synthesize functional nanoparticles has been of great interest recently (Philip, 2009) [8]. The ability of microorganisms to convert oxidation state of metals and their microbial processes has opened up new opportunity to explore novel property i.e biosynthesis of metal nanomaterials. Survival of bacterial diversity in the stress condition leads to develop the new strains which have new properties (Abhilash, 2010) [2]. This survival strategy called as "resistance for metals" by microbes. Microbes have the capacity to alter their environment by selective interaction with metals. The ability of microorganisms to change oxidation state of metals and their microbial processes has opened up new opportunity to explore novel applications such as biosynthesis of metal nanomaterials. These have been successfully synthesized by microorganisms such as actinomycetes, bacteria, fungi and yeast (Slawson *et al.*, 1992) [12]. Keeping view the above advantage of bacteria, bacterial diversity is explored for fabrication of copper nanoparticles.

Material and Methods
Sample collection from selected sites
Twenty two samples in the form of soil, water and pebbles from different sites of Chambaghat, Sataun, Seond and Larji in state of Himachal Pradesh and Khetri Nagar in Rajasthan with an altitude 30º92':77'09', 30º55':77'63', 31º95':77'10' and 28º07':75'82' msl respectively were collected and kept at 4 °C.

Standardization of varous parameyrs for isotation of putative copper nanoparticles synthesizing bacteria
Medium for isolation Tryptone Yeast medium (10g/l Tryptone, 5g/l Yeast Extract, 40mg/l $CuSO_4$, pH: 7.2; Abo-State and Partila, 2015) [1], Nutrient agar (5g/l Peptone, 3g/l Beef extract, 5g/l Sodium chloride, 20g/l Agar, pH: 7.0; Taran *et al.*, 2017) [13], Luria Bertain medium (10g/l Peptone, 5g/l Yeast extract, 10g/l NaCl, pH: 7.2; Ramanathan *et al.*, 2011) [9],

Muller Hinton Agar(Beef extract 2g/l, casein hydrolysate 17.5g/l, starch 1.5g/l, agar 17g/l, pH 7.0;Ghorbani *et al*., 2015) [4] and Bile Esculin Azide Agar (Beef extract 5g/l, casein hydrolysate 17g/l, proteose peptone 3g/l, oxgall 10g/l, esculin 1g/l, ferric ammonium citrate 0.5g/l, sodium chloride 5g/l, sodium azide 0.15g/l, agar 15g/l, pH 7.1; Ashajyothi *et al*., 2014) [3] medium were investigated for producing maximum bacterial colonies (cfu/g/ml) was selected for further isolation of putative copper nanoparticles synthesizing bacteria.

Parameters

Effect of incubation time investigated in the range of 0-120hrs, incubation temperature in the range of 20-50 ºC and pH of medium in the range of 4.0-11.0 on isolation of putative copper nanoparticles synthesizing bacteria.

Isolation of putative copper nanoparticles synthesizing bacterial isolates from collected samples

Enumeration and isolation of bacterial population from each sample was carried out using serial dilution technique which involved dilution of each sample upto 10^{-10}. One gram or ml of each of soil/pebble/water sample was transferred into sterile blanks with final volume 9.0 ml each, which was vigorously shaken using vortex mixer. From this tube 0.1 ml was transferred to next 9.9 ml tube diluting the sample 10 times and in this way dilutions were carried out upto 10^{-10}. Simultaneously five petridishes containing 15-20 ml solidified nutrient agar medium were taken and marked as 10^{-1}, 10^{-3}, 10^{-5}, $10^{-7}10^{-9}$ and 10^{-10}. To each of these petridishes 1.0 ml of sample from each corresponding tube was transferred and was spread all over the agar surface using sterile L-shaped glass rod. All the plates were incubated at an optimum temperature, for optimum incubation time. Mixed populations of different bacteria were found in form of different colony morphotypes, each of the colony morphotype was transferred to 10 ml of selected broth to obtain axenic culture of each bacteria at optimum pH, incubation time and temperature followed by streaking of each axenic culture on selected solidified plates.

Quantitative screening of bacterial isolates for copper nanoparticles synthesizing capability

All bacterial isolates obtained were quantitatively assessed for their ability to synthesize copper nanoparticles using 1% inoculum (overnight culture) of each bacterial isolates by inoculating into 50 ml nutrient broth followed by incubation at 37 ºC for 24 hrs at 150 rpm**.** Supernatant of each bacterial culture was collected by centrifugation at a speed of 8500 rpm (Remi centrifuge) for 15 minutes at 4 ºC to investigate extracellular synthesis of copper nanoparticles. An aqueous solution of $CuSO_4$ ranging from 2 mM was treated with 50 ml of bacterial supernatant in 250 ml Erlenmeyer flask. The whole mixture was incubated at temperature ranging from 37 °C at 150 rpm for 0-72 hrs. Formation of copper nanoparticles was indicated by the colour change of the solution followed by measuring the O.D at 300-800nm wavelength.

Morphological and biochemical characterization (Kristjansson *et al*., 1986)

All selected copper nanoparticles synthesizing bacterial isolates obtained in previous experiment were further studied for various morphological characters which included the colour, shape, size, margin, elevation, surface, pellicle formation and microscopic characters included gram reaction, shape, arrangement, spore formation. The selected copper nanoparticles synthesizing bacterial isolates were further examined for different biochemical reactions *viz*., catalase, malonate, voges-proskauer, citrate, ONPG (O-Nitrophenyl-β-D Galactopyranoside), nitrate reduction, arginine, sucrose, mannitol, glucose, arabinose, and trehalose tests using Rapid KB013 HiBacillus TM Identification Kit.

Analysis of biochemical characters: Biochemical characters of selected bacterial isolates were studied and the data matrices were analysed by the SAHN module of NTSYS-pc version 2.20 and similarities between isolates were estimated using the Jaccard's coefficient, calculated as J= A/ (N-D), where A is the number of positive matches, D is the number of negative matches and N is the total sample size including both the number of matches and unmatches (Rohlf and Milligan, 1994) [10].

Result and Discussion

Sample collection

Copper rich sites located in Sataun village of district Sirmour. Chambaghat village of district Solan, Seond and Larji village of Kullu district of Himachal Pradesh and a copper mine of Khetri Nagar town in Jhunjhunu district of Rajasthan were surveyed and selected for sample collection. A total of twenty two samples in form of soil, pebbles and water from selected copper rich sites were collected. Water samples were collected using a 30ml sterilized syringe whereas soil and pebbles samples were collected using sterile foreceps in flat bottom tubes. All these samples were kept at 4 °C in refrigerator.

Standardization of varous parameyrs for isotation of putative copper nanoparticles synthesizing bacteria

Out of the five medium nutrient broth/agar medium was found to be the best isolation medium as maximum value of growth O.D. of 1.780 at a wavelength of 540 nm was obtained along with maximum number of colonies as compared to other four media. Optimum incubation time was found to be 24 hrs and incubation temperature was found to be 37 °C for isolation of putative copper nanoparticles synthesizing bacteria from samples as it produced a maximum growth. The pH value of 7.0 was found to be optimum for isolation of putative copper nanoparticles synthesizing bacteria. With deviation from neutral pH both on acidic as well as alkaline values growth OD decreased significantly (Fig 1, 2 & 3).

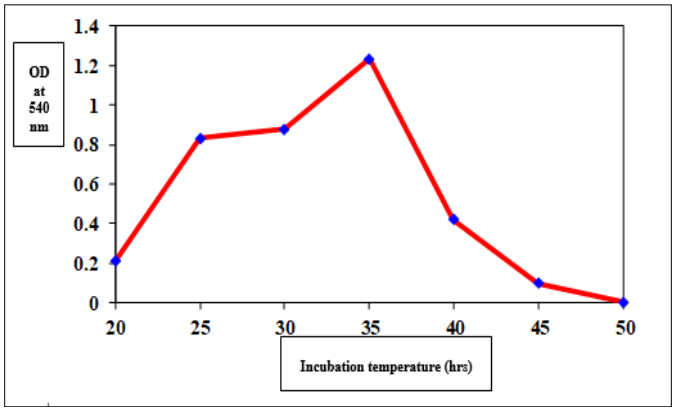


Fig 1: Effect of incubation temperature on growth for isolation of putative copper nanoparticles synthesizing bacteria

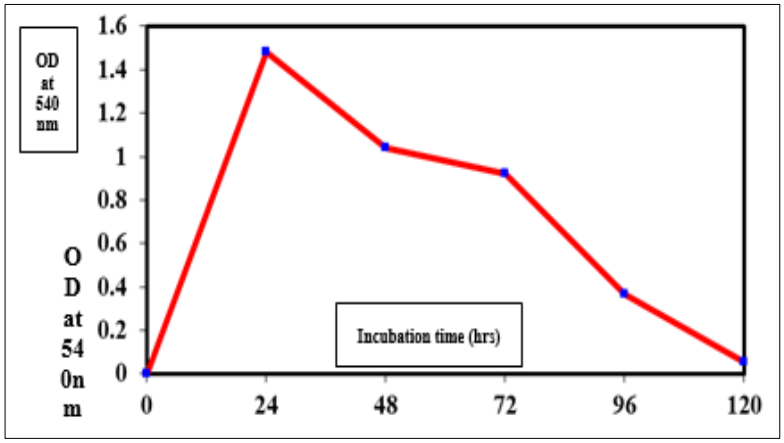


Fig 2: Effect of incubation time on growth for isolation of putative copper nanoparticles synthesizing bacteria

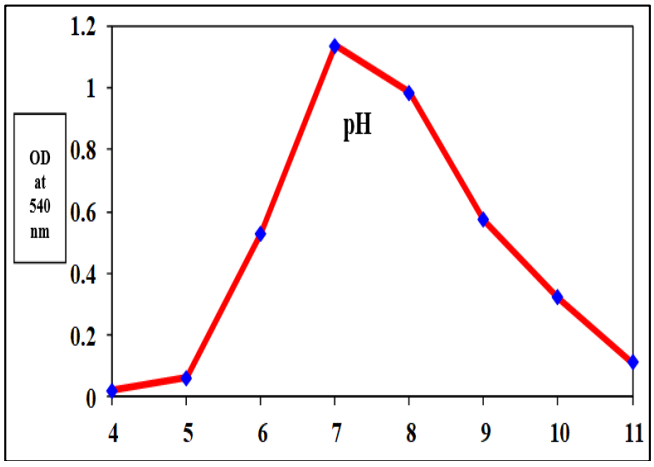


Fig 3: Effect of pH on growth for isolation of putative copper nanoparticles synthesizing bacteria

Isolation of putative copper nanoparticles synthesizing bacterial isolates from collected samples

A total of 154 putative copper nanoparticles synthesizing bacterial isolates were obtained from various selected sites of Himachal Pradesh and Rajasthan using nutrient agar (pH 7.0) at incubation temperature of 35 °C for incubation time period of 24 hrs. Bacterial population of various collected samples was enumerated after 24 hrs of incubation on nutrient agar medium at 35 °C using serial dilution technique. Bacterial population of Chambaghat site ranged from a minimum of 2.21×10^5 cfu/g/ml to a maximum of 5.12×10^5 cfu/g/ml. In case of Sataun site bacterial population ranged from a minimum of 2.12×10^5 cfu/g/ml to a maximum of 4.80×10^5 cfu/g/ml. At Seond site bacterial population ranged from a minimum of 1.42×10^5 cfu/g/ml to a maximum of 4.16×10^5 cfu/g/ml whereas in Larji site bacterial population ranged from a minimum of 2.26×10^5 cfu/g/ml to a maximum of 3.28×10^5 cfu/g/ml. Finally at Khetri Nagar site minimum bacterial population of 3.00×10^5 cfu/g/ml and maximum of 4.02×10^5 cfu/g/ml was observed (Table 1).

Table 1: Enumeration of bacterial population of various samples from selected sites

Sample sites	Bacterial population on nutrient agar after 24 hrs of incubation (cfu/g/ml)				
Chambaghat					
Soil		Water		Pebbles	
S1	5.12×10^5	W1	2.46×10^5	P1	2.24×10^5
S2	4.22×10^5	W2	2.38×10^5	P2	2.21×10^5
Sataun					
S1	4.80×10^5	W1	2.12×10^5	-	
S2	3.48×10^5	W2	2.84×10^5	-	
Seond					
S1	4.16×10^5	W1	2.62×10^5	-	
S2	3.00×10^5	W2	1.42×10^5	-	
Larji					
S1	3.28×10^5	W1	2.38×10^5	-	
S2	2.60×10^5	W2	2.26×10^5	-	
Khetri Nagar					
S1	4.02×10^5	W1	3.12×10^5	-	
S2	3.64×10^5	W2	3.00×10^5	-	

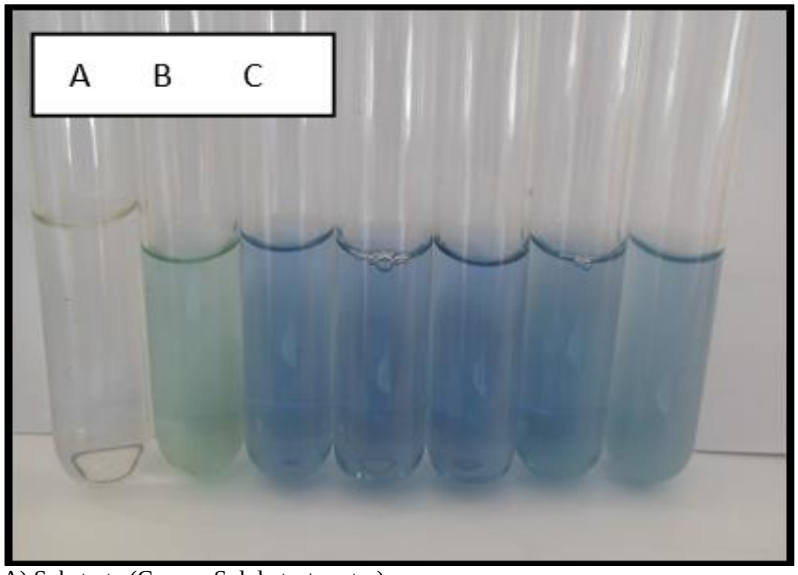
The intracellular mechanism consists of transporting ions into the microbial cell to form nanoparticles in the presence of enzymes. Whereas extracellular synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes. In the present study, for bioprospecting of copper nanoparticles synthesizing bacteria, sites of Chambaghat village of Solan districts, Sataun village of Sirmour districts and Seond and Larji villages of Himachal Pradesh and one Khetri Nagar site of Rajasthan were investigated. Kaur *et al.* (2015) [6] lso reported isolation of copper nanoparticles synthesizing bacteria *Kocuria flava* from marine sediment collected from Kanyakumari coast. Tiwari *et al.* (2016) [14] were also successful for isolation of copper nanoparticles synthesizing *Bacillus cereus* from soil collected from copper mining area of Malanjkhand Copper Project (Madhya Pradesh) and Khetri Copper Complex (Rajasthan).

Quantitative screening of bacterial isolates for copper nanoparticles synthesizing capability

A total of 154 bacterial isolates obtained, were screened individually for their ability to synthesize copper nanoparticles. One percent concentration of inoculum (overnight culture) was inoculated into nutrient broth for copper nanoparticles synthesis followed by incubation at 35 °C for 24 hrs at 150 rpm. The supernatant was collected by centrifugation at 8500 rpm, 4 °C for 15 mins to investigate synthesis of copper nanoparticles. Ten ml of each supernatant obtained was mixed with 10 ml of 2.0 mM solution of copper sulphate ($CuSO_4$) solution and incubated at 35 °C upto 72 hrs. Formation of copper nanoparticles was indicated by colour change of the solution from light blue to greyish blue (Plate 1) which was further confirmed by measuring its O.D. value at a wavelength of 600 nm. It was observed that maximum synthesis occurred from 24-48 hrs at 35 °C. Whereas decline in activity has been observed upto 72 hrs.

In present study copper nanoparticles synthesizing activity ranged from a minimum of 0.001 to a maximum of 0.489 in 49 bacterial isolates obtained from Chambaghat site of Solan district. In case of 31 bacterial isolates obtained from Sataun site of Sirmour district copper nanoparticles synthesizing activity ranged from a minimum of 0.001 to a maximum of 0.460. In Seond site of kullu district copper nanoparticles synthesizing activity of 24 bacterial isolates ranged from a minimum of 0.001 to a maximum of 0.427 and all the 21 bacterial isolates of Largi site of Kullu district copper nanoparticles synthesizing activity ranged from a minimum of 0.034 to a maximum of 0.375. Whereas 29 bacterial isolates obtained from Khetri nagar site of Rajasthan copper nanoparticles synthesizing activity ranged from a minimum of 0.020 to a maximum of 0.432.

Quantitative screening of all 154 bacterial isolates for copper nanoparticles synthesizing ability was examined successfully using 2.0 mM copper sulphate solution. Similar reports of use of copper sulphate have been found in literature for copper nanoparticles synthesis by *Serratia* sp. (Hasan *et al*., 2007) [5], *Morganella morganii* RP4 and *Morganella psychrotolerans* (Ramanathan *et al*., 2009) [9], *Enterococcus faecalis* (Ashajyothi *et al*., 2014) [3], *Pseudomonas flouresences* (Shantkriti and Rani, 2014) [11], *Bacillus cereus* (Tiwari *et al.,* 2016) [14] and *Bacillus* sp. (Taran *et al*., 2017) [13]. Whereas other authors have reported use of copper nitrate for synthesis of copper nanoparticles by *Kocuria flava* (Kaur *et al.*, 2015) [6] and *Salmonella typhimurium* (Ghorbani *et al.,* 2015). In present study synthesis of copper nanoparticles was confirmed by colour change of the solution from light blue to greyish blue (Plate 1). Other authors have also reported change in colour of solution from light blue to dark green depicting synthesis of copper nanoparticles by *Morganella morganii* RP4 and *Morganella psychrotolerans* (Ramanathan *et al*., 2009) [9], *Pseudomonas fluresences* (Shantkriti and Rani, 2014) [11], *Bacillus cereus* (Tiwari *et al*., 2016) [14] and *Bacillus* sp. (Taran *et al*., 2017) [13]. Whereas *Salmonella typhimurium* (Ghorbani *et al.,* 2015) [11] produced cloudy orange color indicated formation of copper nanopartciles.



A) Substrate (Copper Sulphate + water)
B) Uncultured nutrient broth+Substrate
C) Supernatant of bacterial culture + Substrate (Copper nanoparticles suspension)

Plate 1: Colour change of solution from light blue to greyish blue after 24 hrs. Incubation period

Morphological and biochemical characterization

Colour of colonies exhibited by 154 bacterial isolates was found to vary from white, cream, yellow, pale yellow, brown, pink and transparent. 12.99% were found to be white, 64.93% were found to be cream, 7.79% were yellow, 6.49% were transparent, 3.89% were pale yellow, 3.24% were pink whereas only 0.64% were found to be brown in colour. Among these isolates 20.12% produced circular shaped colonies while 79.87% isolates were found to produce irregular shaped colonies. Among these isolates majority of 79.22% were found to produce smooth colonies while 20.77% possessed rough surfaced. Colonies of 3.89% bacterial isolates were found to be raised whereas 96.10% isolates were found to possess flat elevation. Colonies of 20.12% bacterial isolates were found to possess entire margins, 77.27% possessed undulated margins and 2.59% showed fili form margins. Out of 154 bacterial isolates 25.32% were found positive and 74.67% were negative for pellicles formation.

Out of a total of 154 copper nanoparticles synthesizing bacterial isolates, 30 bacterial isolates obtained from all four sites of Himachal Pradesh and one site of Rajasthan were selected for biochemical characterization which possessed maximum copper nanoparticles synthesizing activity in the range of 0.001-0.489 after 24-48 hrs. Out of which 50%

bacterial isolates were found positive for catalase test, 23% isolates were positive for arginine test, 20% isolates were positive for malonate, 40% were positive for Voges-Proskauer test, 43.33% were positive for citrate test, 13.33% were positive for ONPG (O-Nitrophenyl-β-D Galactopyranoside) test, 86.66% were found positive for nitrate reduction test. Bacterial isolates showed significant variation for the utilization of various carbon sources *viz.*, glucose, sucrose, mannitol, arabinose and trehalose. Glucose was fermented by 80.00% copper nanoparticles synthesizing bacterial isolates whereas sucrose was fermented by 53.33% of the total copper nanoparticles synthesizing bacterial isolates. It has been found that mannitol, arabinose and trehalose sugars were fermented by 30.00%, 6.66%, and 70.00% copper nanoparticles synthesizing bacterial isolates, respectively.

Results obtained from biochemical characterization of all selected 30 bacterial isolates were analysed by the SAHN module of NTSYS-Pc version 2.20 and dendrogram was constructed using UPGMA. According to the biochemical characters dendrogram were constructed which showed that, dendrogram bifurcates into two clusters A and B, cluster B clearly separates Rajasthan water isolate RKW2.1. from other 29 copper nanoparticles synthesizing bacterial isolates. Cluster A further sub divided into 2 group C and D where D group clearly shown that Kullu Largi soil isolate KLS1.4 different from the isolates of group C. Cluster C further subdivided into 2 group where isolates of one group shows 30% similarity with another group. Further dendrogram clearly shown that there were three groups shown 100%. In one group KSW1.1, KSW2.4 and KLW2.5 bacterial isolates shown 100%, in second group KLS1.1, RKS1.3 and RKS1.4 were showing 100% similarity and in another group SCS1.1 and SCS1.2 bacterial isolates were also shown 100% similarity. The dendrogram constructed based on results of biochemical characters showed that all the bacterial isolates possess only 24.0% similarity to one another depicting significant variation/dissimilarity among thirty copper nanoparticles synthesizing bacterial isolates (Fig. 4). Persual of literature one report shown that maximum isolated copper resistant bacteria were gram positive (Tiwari *et al*., 2016) [14]. Both gram positive and negative bacteria have ability to synthesize copper nanoparticles. *Enterococcus faecalis* (Ashajyothi *et al*., 2014) [3], *Kocuria flava* (Kaur *et al*., 2015) [6], *Bacillus cereus* (Tiwari *et al*., 2016) [14] and *Bacillus* sp. FU4 (Taran *et al*., 2017) [13] were gram positive bacteria having the ability to synthesize copper nanoparticles whereas *Serratia* sp. (Hasan *et al*., 2007) [5] and *Pseudomonas fluorescens* (Shantkriti and Rani, 2014) [11], were gram negative bacteria also having the ability to synthesize copper nanoparticles.

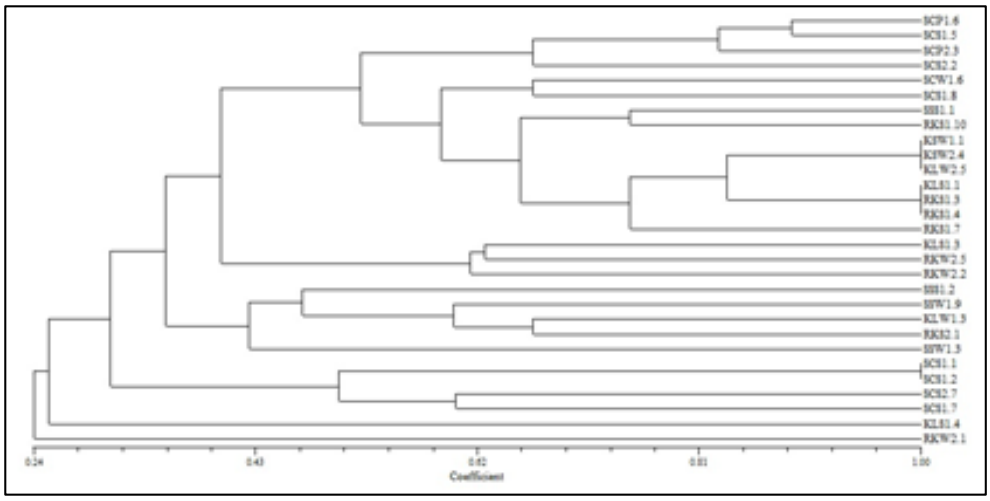


Fig 4: Dendrogram showing relatedness among selected copper nanoparticles synthesizing bacterial isolates based on biochemical characters

Conclusion

In the present study, 154 bacterial isolates were characterized initially on the basis of different colony and microscopic characters and 30 selected bacterial isolates were further characterized by biochemical characters and it was giving us an idea that more than 50% copper nanoparticles synthesizing bacterial isolates belong to genus *Bacillus*. But in comparison to synthesizing maximum copper nanoparticles, gram negative bacteria has great potential to synthesis maximum copper nanoparticles.

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