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Characterization of Isolated Strains of Microorganisms From Mineral, Mountain and Spring Waters from France, Italy, England, South Korea, Japan, Netherlands, Austria, Spain, Singapore, Germany, Switzerland, Greece, Turkey, Dubai and Bulgaria

Nedyalka Valcheva

Dimitrovgrad, Haskovo District, Bulgaria.

***Corresponding author**

Nedyalka Valcheva,
Dimitrovgrad, Haskovo District, Bulgaria.

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Introduction

The aim of the present work is to isolate, identify and investigate the species of bacteria from healing, mountain and spring waters in the area of Paris, France, Rome, Italy, London, England, Busan, South Korea, Tokyo, Japan, Amsterdam, Holland, Vienna and in Bulgaria. 127 bacterial strains were isolated and their colonial and morphological characteristics were determined and the study strains were identified. The data show that the isolated 9 strains from the hypothermal healing spring waters in the Paris area, France were identified as *N. Valcheva - Lisinibacillus pakistanensis*, *Serratia marcescens* (four strains), *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Bacillus pumilus* and *Bacillus cereus*. The isolated strains from thermal healing springs in Saturnia, Tuscany region and Fontana di Trevi in Rome, Italy were identified as *N. Valcheva - Tiobacillus Ferrooxidans*, *Staphylococcus cohnii*, from a hypothermal spring in the London area as *N. Valcheva - Aeromonas caviae*, *Pseudomonas chlororaphis*, from a mountain spring in the area of Busan, South Korea, was identified as *N. Valcheva - Bacillus safensis*, *Staphylococcus cohnii*, *Pseudomonas chlororaphis*, *Staphylococcus cohnii*. From a mountain geothermal spring in the area of Tokyo, Japan, strains were identified as *N. Valcheva - Bacillus safensis*, *Phellodendron chinense*, *Aeromonas caviae*, *Pseudomonas japonica*. The strains identified in the Amsterdam, Netherlands area were identified as *N. Valcheva & A. Atanasova - Aeromonas viridans*, *N. Valcheva & V.P. Panteleev - P.japonica*, *N.Valcheva & V.V.Panteleev - Aeromonas salmonicida*, *N.Valcheva & A.V. Panteleev - Aeromonas caviae*, *N.Valcheva & L.A.V. Panteleeva - Phellodendrom chinense*. Sulfur bacteria of the species *N. Valcheva - Chlorobium chlorochromatii* were identified from the healing springs in the region of Baden and Vienna, Austria. The strains from Bulgaria were identified from 100 hypothermal, thermal and common springs from 11 regions and belong to the following 14 genera: *N. Valcheva - Bacillus*, *Brevibacillus*, *Geobacillus*, *Aeromonas*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, *Stenotrophomonas*, *Serratia*, *Nocardia*, *Trichosporon*, *Azoarens*, *Lisinibacillus*, *Rhodococci*. From the hypothermal salt source in Barcelona,

in the area to the Mediterranean Sea, 29 species of organisms were identified. *N. Valcheva & St.Mihailov - Pseudomonas shengduensis*, *N. Valcheva & V. Marinova - Bacillus cereus* (3 strains), *N. Valcheva & I.D.Valchev - Shevanelia algae* (3 strains), *N.Valcheva & D. I.Valchev - Acinetobacter quillouiae*, *N. Valcheva & N.I.Valcheva - Pseudomonas pseudoalcaligenes*, *N.Valcheva & I.I.Valcheva - Pseudomonas korensis*, *N.Valcheva & P.N.Valcheva - Delftia acidovorans* (2 strains), *N.Valcheva & M.P.Pantelev - Citrobacter freundii*, *N. Valcheva & M.I.Panteleeva - Pseudomonas rhodesiae*, *N.Valcheva & D. Eschkenazy - Pseudomonas Chlororaphis*, *N. Valcheva & H.Eschkenazy - Pseudomonas korecensis*, *N.Valcheva - M.Eschkenazy - Pseudomonas chlororaphis*, *N. Valcheva & G. Dimov - Stenotrophomonas sp.*, *N.Valcheva & M. Naneva - Pseudomonas rhodesiae*, *N.Valcheva & N.Nanev - Enterobacter Ludwigii*, *N. Valcheva & St.Stratiev - Aeromonas caviae* (2 strains), *N.Valcheva & P. Panteleev - Aeromonas mediae*, *N.Valcheva & E. Todorovska - Providencia rettgeri*, *N.Valcheva - A.Stoyanova - Myroides odoratimimus*, *N.Valcheva & R. Moneva - Alcaligenes faecalis*. *N. Valcheva - Bacillus safensis* is the only test that shows a significant reduction in the number of cells by 50% compared to cells in lung cancer A549. Concentration from 100 µg /mL. Metabolomic profiling of crude extracts from *N. Valcheva - Bacillus safensis*, isolated from a source in Tokyo and a source in Busan, South Korea, near the spring of South China Sea, reveals the presence of known antibacterial and/or anticancer agents such as licorin, angustin, krinamidin, Vaccinol and poulin. *N. Valcheva - Acinetobacter guillouiae* (born in Barcelona) degrades plasma. With probiotic action from *N. Valcheva - Bacillus subtilis* and *N. Valcheva - Bacillus methylotrophius* (Bulgaria). *N. Valcheva & I. I.Valcheva - Pseudomonas korensis* is used for vaccination. The identified strains from a source in the Singapore area are: *N. Valcheva - Aeromonas caviae*, *Klebsiella oxytoca*, *Kurthia gibsoni*, *Roatella ornithinolitica*, *Kurthia gibsonii*, *Pseudomonas aeruginosa*, *Citrobacter cloacae*, *Klebsiella pneumoniae*, *Alcaligenes faecalis*, *Aeromonas veronii*, *Serratia marcescens*, *Enterobacter hormchei*, *Enterobacter cloacae*,

Citrobacter freundii, *Debaromyces hansenii*, *Pseudomonas putida*, *Pseudomonas sp.* The identified strains from a spring in the Berlin, Germany area are: N. Valcheva-Klebsiella, pneumoniae, N. Valcheva & A. Dechev, N. Valcheva & T. Bazaitova *Serratia liquafaciens*, N. Valcheva-*Aerococcus oxytoca*, *Buttiauxella gaviniae*, *Debaromyces hansenii*. Identified microorganism from a mineral spring in the Athens region, Greece - N. Valcheva - *Bacillus pumilus*. Identified Microorganism in Dubai, UAE - N. Valcheva - *Bacillus mesentericus*, Identified Microorganism in Alanya, Turkey and N. Valcheva - *Bacillus subtilis* with probiotic effect in mineral springs. All isolated strains have enzyme, anti-fungal and antibacterial activity. Remediation is used to extract valuable metals or remove metals that are harmful to organisms. All strains have different biological activity. The difference in the type of strains identified can be explained by the influence of the composition of the water, the type of soil and rocks through which it passes, the temperature, the pH of the environment and the different geographical location.

Colloidal nanomaterials with antibacterial activity are applied in practice. Most commonly used are colloidal Nano silver (Ag) and copper (Cu) as separate nanomaterials, and combinations between Ag and Cu with different concentrations in ppm. Studies are performed of colloidal nano solutions with two and four types of nanoparticles. The first two solutions are with colloidal nano silver (Ag) and Copper (Cu). The third solution is with colloidal nano silver (Ag), copper (Cu), zinc (Zn), silicon (Si). Effects are studied over the following bacteria - *Escherichia coli*, *Enterococci* and *Coliforms*. The studied colloidal solutions can be used to disinfect surfaces and neutralize bacteria in wastewater.

And to determine the Microbiological Effects of Combinations of Colloidal Silver (Ag), Copper (Cu), Silicon (Si) and Zinc (Zn) Nanoparticles.

According to the European Environment Agency the wastewater must not contain pathogenic microorganisms. Their purification can be by physical, chemical or biological means. The aim is to remove pollutants that are dangerous to humans from sewage and industries. Different technologies are utilized – filtration, anaerobic treatment with antagonistic bacteria (e.g. *Bacillus subtilis*), and other organisms. The wastewater treatment is done in order to ensure subsequent reuse of water. The treated wastewater is mostly used in agriculture. This protects the soil from contamination with harmful substances, acidification, salinisation, surface over wetting and erosion during irrigation. It is necessary to flow purified water into the rivers without causing water pollution. By using wastewater in the agriculture, more freshwater resources can be provided for other purposes, including nature and households. If the quality of regenerated water is managed properly, the purified wastewater can provide an effective alternative that can meet the water needs of agriculture. Most common in sewage and industrial wastewater are *Escherichia coli*, *Enterococci* and *Coliforms*, which can cause infectious diseases. Over the last 30 years' preparation of solutions with

antibacterial effects has been developing in global application science. Most importantly the solutions for wastewater treatment must be durable over time. Experience shows that solutions containing positive ions last in time. They absorb the waste products, and they are precipitated and separated in treatment systems. There are different solutions with positively charged ions. In electrochemically activated water Anolyte, we have acidic medium with pH=2.5-3.5 and oxidation reduction potential (ORP) (+600–+800) mV. The effects are mainly due to hydrogen ions (H⁺) and oxidants Cl₂O, ClO₂, ClO⁻, HClO, Cl₂, O₂, O₃, HO₂ (Bachir & Pogorelov, 2018; Lata et al., 2016; Dube & Jain, 2018; Popova et al., 2018; Popova et al., 2019).

In the anolyte is shown formation of clusters of 20-22 water molecules with size 0.822 nm at (-0.1212 eV) ($\lambda=10.23 \mu\text{m}$) ($\tilde{\nu}=978 \text{ cm}^{-1}$) with anti-inflammatory and antibacterial effects (Ignatov, et al., 2021). Effects are demonstrated for *Escherichia coli*, *Enterococci* and *Coliforms* at (-0.1212 eV) ($\lambda=10.23 \mu\text{m}$) ($\tilde{\nu}=978 \text{ cm}^{-1}$) (Gluhchev et al., 2015). Antibacterial effects with *Escherichia coli*, *Enterococci* and *Coliforms* have illustrated in study from South Africa (Kirkpatrick, 2011). The local extremum at (-0.1212 eV) ($\lambda=10.23 \mu\text{m}$) ($\tilde{\nu}=978 \text{ cm}^{-1}$) was expressed in the research with colloidal nano silver and combination of colloidal nano silver and copper (Ignatov et al., 2020). In the research was applied the spectral analyses with method NES (Gramatikov et al., 1992; Antonov, 1995; Todorov et al., 2018).

Polysaccharides extracted from natural sources have attracted extensive attention in recent years. There is local extremum at (-0.1212 eV) ($\lambda=10.23 \mu\text{m}$) ($\tilde{\nu}=978 \text{ cm}^{-1}$) in the spectrum of polysaccharides (Zhbakov et al., 1995; Cheng et al., 2011). There are natural polysaccharides with anti-inflammatory effects (Hou, 2020). There are results at (-0.1212 eV) ($\lambda=10.23 \mu\text{m}$) ($\tilde{\nu}=978 \text{ cm}^{-1}$) with *Lonicera japonica* Thunb (Yan et al., 2016). *Moringa oleifera* Lam., *Urtica dioica* L., *Malva sylvestris* L., *Plantago major* L. (Ignatov et al., 2021).

The local extremum at (-0.1262 eV) ($\lambda=9.82 \mu\text{m}$) ($\tilde{\nu}=1018 \text{ cm}^{-1}$) is expressed in the plant *Scutellaria indica* L. (Cuong et al., 2015).

The local extremum at (-0.1262 eV) ($\lambda=9.82 \mu\text{m}$) ($\tilde{\nu}=1018 \text{ cm}^{-1}$) was expressed in the research of combination of colloidal nano silver and copper (Ignatov et al., 2020). The study shows this local extremum with anti-inflammatory effect with colloidal particles (Mohana & Sumathi, 2020).

We have the following reaction in colloidal nano silver (AgNPs) (Sun et al., 2020).



This process is due to the properties of polar water molecules to interact with each other by intermolecular dipole-dipole interactions and hydrogen bonds. As a result, H₂O molecules are formed around Ag⁺ ions the hydrated aqueous layer, capable of screening off oppositely charged ions from the interaction, that is why those ions acquire higher stability in aqueous solutions.

For the research is used wastewater with presence of following bacteria –*Escherichia coli*, *Enterococci* and *Coliforms*. Achieved are results for purification of drinking waters from *Escherichia coli* (Bilal et al., 2019). Colloidal Nano Silver has antibacterial properties for *Escherichia coli*, *Enterococci* and *Coliforms* (Feng et al., 2021; Popova et al., 2021). Studies show that with the increase the concentration of colloidal nanoparticles is increased the effect. For *Escherichia coli*, *Enterococci* and *Coliforms* there are proven differences at 20 and 30 ppm. With the increase of ppm, the effect increases (Popova & Ignatov, 2021). For SARS-CoV-2, it is demonstrated how the increase of concentration from 20 to 100 ppm leads to cumulated effect (Jeremiah et al., 2020). AgNPs is applied with antibacterial effect for food packaging (Carbone et al., 2016; Simbine et al., 2019; Balandin et al., 2015). Colloidal Nano Silver has practical application in medicine. Examinations indicate that AgNPs reduces the risk of secondary microbial infections in patients with COVID-19 because they inhibit bacteria and fungi, which can pollute healthcare facilities (Vazquez-Munoz & Lopez-Ribot, 2020).

The effect of eye drops is stabilized (Concepcion et al., 2007). Antibiotic activity is demonstrated in Gram-Negative and Gram-Positive Bacteria (Domínguez et al., 2020). Colloidal Nano Silver and Copper has surface disinfection effects (Talebian et al., 2020; Kampf, 2020). There are in vitro effects of colloidal nano copper (Popova, et al., 2004).

Methods

Applied is a mixture of wastewater with colloidal (v) solution in proportion 50-50 (v/v %). These proportions are tested in colloidal solutions with different solvents (Yola et al., 2014; Valcheva et al., 2020). The purpose of current research is to perform microbiological analyses of two colloidal solutions with Cu, Ag, and a third one with Cu, Ag, Zn, Si. The study is conducted for wastewater with *Escherichia coli*, *Enterococci* and *Coliforms*.

Device for Colloidal Nanoparticles

For the preparation of two colloidal solutions with Cu, Ag, and a third one with Cu, Ag, Zn, Si is used a patented device. Details of patent for Patent Application: PCT/EP2021/054691 (Patent PCT/EP2021/054691, 2001). 25.02.2021. Title: Control device and method for driving electrodes of at least one electrolysis device for electrochemical production of nanoparticles. Description: The invention relates to the technical field of water treatment. The object of the invention is a control device and a method for driving electrodes of at least one electrolysis device for the electrochemical production of nanoparticles in water according to the generic terms of claims 1 and 10.

Concentration of EVODROP Silver Nanoparticle in Sample with Bacteria

1. First sample with 500 mL – 500 mL Ag 20 Cu 20 ppm; 500 mL water from control sample with bacteria;
2. Second sample with 500 mL – 500 mL Cu 20 Ag 30 ppm; 500 mL water from control sample with bacteria;
3. Third sample with 500 mL – 500 mL Cu 30 Ag 30 Zn 22 Si 22 ppm 500 mL water from control sample with bacteria;

In the materials and methods are included nutrient media and methods for determination of microbiological indicators.

Nutrient Media

1. Nutrientagar (MPA) withcontents (in %) – meatwater, peptone – 1%, agar –agar – 2%. Endo'sMedium (fordefining of *Escherichiacoliandcoliformbacteria*) withcontents (g/dm³) – peptone– 5.0; triptone– 5,0; lactose – 10.0; Na₂SO₃ – 1.4; K₂HPO₄– 3.0; fuchisine– 0.14; agar – agar– 12.0; pH 7.5 – 7.7.
2. Nutrientgelatine (MPD) (fordefining of *Pseudomonasaeruginosa*) withcontents (in%) – Peptidigest of animaltissue; 25 % gelatin; pH = 7.0 – 7.2.
3. Mediumfordefining of enterococci (esculin – bileagar).
4. Medium for defining of sulphite reducing bacteria (Iron Sulfite Modified Agar).
5. Wilson-Bleermedium (for defining of sulphite reducing spore anaerobes (*Clostridium perfringens*) with contents (g/dm³) – 3% Nutrient agar; 100 cm³ 20% solution Na₂SO₃; 50 cm³ 20% glucosesolution; 10 cm³ 8% solution of Fe₂SO₄.

Methods for Determination of Microbiological Indicators

1. Methods for evaluation of microbiological indicators according to Ordinance No. 9 / 2001, Official State Gazette, issue 30 and decree No.178/23.07.2004 about the quality of water, intended for drinking purposes.
2. Method for determination of *Escherichia coli* and *coliform* bacteria – BDS EN ISO 9308 – 1: 2004;
3. Method for determination of enterococci – BDS EN ISO 7899 – 2;
4. Method for determination of sulphite reducing spore anaerobes – BDS EN 26461 – 2: 2004;
5. Method for determination of total number of aerobic and facultative anaerobic bacteria – BDS EN ISO 6222: 2002;
6. Method for determination of *Pseudomonas aeruginosa* – BDS EN ISO 16266: 2008.
7. Determination of coli – titre by fermentation method – Ginchev's method
8. Determination of coli – bacteria over Endo's medium – membrane method.
9. Determination of sulphite reducing anaerobic bacteria (*Clostridium perfringens*) – membrane method.

Results

Microbiological Indicators of Control Sample

For the research of effects of nanoparticles was studied control sample with bacteria. Table1 illustrates the average results of control sample with 10 measurements of the microbiological indicators after 24 and 36 hours of the following bacteria – *Escherichia coli*, *Enterococci* and *Coliforms*. The result is with wastewater.

Table 1: Result with wastewater as control sample with data for *Escherichia coli*, *Enterococci* and *Coliforms*.

Controlled parameter	Limit value, cfu/cm ³	Result, cfu/cm ³
Coliforms	0/100	169
<i>Escherichia coli</i>	0/100	169
<i>Enterococci</i>	0/100	19
Total number of microorganisms at 22 °C	100	180
Total number of microorganisms at 37°C	20	169

The results show that the tested water is not suitable for drinking purposes according to Ordinance No. 9 /2001, Official State Gazette, issue 30, and decree No. 178 / 23.07.2004 about the quality of water, intended for drinking purposes. The controlled parameters are defined by the membrane method, and by using of differential diagnostic nutrient media at 24 and 36 hours.

For the research of effects of nanoparticles was studied a control sample with bacteria.

Samples For Research

The following samples are used for studying the effect of colloidal solutions of patent (Simbine et al., 2019). when mixing with wastewater:

1. First sample with 500 mL – 500 mL Cu 20Ag 20 ppm (mg/ m³); 500 mL water from control sample with bacteria;
2. Second sample with 500 mL – 500 mL Cu 20 Ag 30 ppm; 500 mL water from control sample with bacteria;
3. Third sample with 500 mL – 500 mL Cu 30 Ag 30 Zn 22 Si 22 ppm 500 mL water from control sample with bacteria;

T-test of Student was applied with 10 measurements of effects on samples of Ag 20 Cu 20 ppm with wastewater. The control tested samples were of wastewater. Statistically significant difference between the two observed groups has been identified, watching closely the effects according to the t-test of Student at level $p < 0, 01$.

Table 2: shows microbiological analysis of Ag 20 Cu 20 ppm water from control sample with bacteria.

Controlled parameter	Limit value, cfu/cm ³	Sample Ag 20 Cu 20 ppm	Result, Control Sample cfu/cm ³
<i>Coliforms</i>	0/100	0	169
<i>Escherichia coli</i>	0/100	0	169
<i>Enterococci</i>	0/100	0	19
Total number of microorganisms at 22 °C	100	0	180
Total number of microorganisms at 37°C	20	0	169

T-test of Student was applied with 10 measurements of effects on samples of Ag 20 Cu 30 ppm with wastewater. The control tested samples were of wastewater. Statistically significant difference between the two observed groups has been identified, watching closely the effects according to the t-test of Student at level $p < 0, 05$.

Table 3: illustrates microbiological analysis of Ag 20 Cu 30 ppm water from control sample with bacteria.

Controlled parameter	Limit value, cfu/cm ³	Sample Ag 20 Cu 20 ppm	Result, Control Sample cfu/cm ³
<i>Coliforms</i>	0/100	0	169
<i>Escherichia coli</i>	0/100	0	169
<i>Enterococci</i>	0/100	0	19
Total number of microorganisms at 22 °C	100	1	180
Total number of microorganisms at 37°C	20	1	169

T-test of Student was applied with 10 measurements of effects on samples of Cu, Ag 30 Si, Zn 22 ppm with wastewater. The control tested samples were of wastewater. Statistically significant difference between the two observed groups has been identified, watching closely the effects according to the t-test of Student at level $p < 0, 01$.

Table 4: shows microbiological analysis of Cu, Ag 30 Si, Zn 22 ppm and water from control sample with bacteria.

Controlled parameter	Limit value, cfu/cm ³	Sample	Result, Control Sample cfu/cm ³
<i>Coliforms</i>	0/100	0	169
<i>Escherichia coli</i>	0/100	0	169
<i>Enterococci</i>	0/100	0	19
Total number of microorganisms at 22 °C	100	0	180
Total number of microorganisms at 37°C	20	0	169

The given results in tables 1, 2 and 3 demonstrate effects of colloidal solutions by patented device (Simbine et al., 2019). For practical purposes, it is necessary to calculate the minimum values for effects in wastewater. The studied colloidal solutions are applicable for surface disinfection.

Conclusions

Conducted are microbiological examinations of the following solutions:

1. First sample with 500 mL – 500 mL Ag 20 Cu 20 ppm; 500 mL water from control sample with bacteria;
2. Second sample with 500 mL – 500 mL Cu 20 Ag 30 ppm; 500 mL water from control sample with bacteria;
3. Third sample with 500 mL – 500 mL Cu 30 Ag 30 Zn 22 Si 22 ppm 500 mL water from control sample with bacteria;

Statistically credible results are obtained. The resulting solutions by technology (Simbine et al., 2019). can be applied for surface disinfection. In the case of wastewater, it is necessary to do applied projects for specific circumstances.

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