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IDENTIFICATION OF LACTOBACILLUS IN CHEDDAR CHEESE SAMPLES AND THEIR APPLICATION IN THE SYNTHESIS OF SILVER NANOPARTICLES FROM SILVER NITRATE

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ABSTRACT

Probiotics are living microorganisms having more beneficial effects on human health when ingested in adequate amounts. Consequently, probiotics are being applied in the food industry where Lactobacillus species are widely used. Hence, this study was aimed to identify Lactobacillus in five cheddar cheese samples, synthesis of silver nanoparticles (AgNPs) using Lactobacillus and to analyze antibacterial properties of synthesized AgNPs. Five samples were cultured on MRS agar. Lactobacillus was identified by colony morphology and biochemical assays. AgNPs were synthesized using identified Lactobacillus. The absorbance of synthesized AgNPs was measured by UV-Visible spectroscopy and the size of the nanoparticles was evaluated by scanning electron microscope (SEM). Antibacterial activity of synthesized AgNPs and isolated Lactobacillus cell-free suspension was evaluated by well diffusion assay. All biochemical assays confirmed the presence of Lactobacillus in four-cheese samples (gram-positive bacilli, catalase-negative, non-acid fast, and non-spore-forming bacteria). UV-Visible spectra showed multiple absorbance peaks at a range of 390-450nm confirming the formation of AgNPs. The SEM analysis indicated well-dispersed agglomerates of spherical shape AgNPs with size range from 50-100nm and the presence of Lactobacillus. A significant difference (P-value < 0.05) was observed in the antibacterial activity of AgNPs and Lactobacillus cell-free suspension against Staphylococcus aureus and Escherichia coli. Furthermore, cell-free suspension showed a significantly high inhibitory effect on Staphylococcus aureus while AgNPs showed a high inhibitory effect against Escherichia coli (P-value < 0.05). Thus, biosynthesized AgNPs serve as a remarkable antibacterial agent to treat infectious diseases caused by multidrug-resistance pathogens which have become a global crisis and will result in great potential in nanomedical applications.

Keywords: Probiotics, Cheese, Lactobacillus, AgNPs, Antibacterial activity

INTRODUCTION

Introduction to probiotics

Probiotics are living microorganisms confer positive health effects, on the host when ingested in adequate amounts (Somashekaraiah et al., 2019). Probiotics have been significantly associated with the immune, digestive, and respiratory system. Furthermore, they modulate immune and digestion function and maintain proper balance in the gut microbiome consequently prevent gastrointestinal disorders including diarrheal diseases, necrotizing enterocolitis, lactose intolerance (Patrignani et al., 2019). Many types of bacteria are considered to have probiotic properties. However, Lactic acid bacteria

(LAB) and Bifidobacteria are the most common groups (Mulaw et al., 2019).

Lactobacillus as probiotic bacteria and their significance in host health

Lactobacillus is a gram-positive, rod-shaped, non-spore-forming, catalase-negative bacteria, capable of producing lactic acid as the primary end product of carbohydrate fermentation (Chakraborty and Bhowal, 2015). Lactobacillus naturally dominates the human vaginal microbiota, skin, gastrointestinal tract, and oral cavity (Pan et al., 2020).

The antibacterial activity of Lactobacillus contributes to many beneficial effects on human health. Currently, several studies have demonstrated that Lactobacillus exhibit antibacterial activity via different mechanisms. Tebyanian et al., (2017) reported that Lactobacilli produce antagonistic substances that inhibit pathogenic microorganisms such as Escherichia coli, Shigella dysenteriae, Salmonella paratyphi A, and Staphylococcus aureus. Results of a study carried out by Shokryazdan et al., (2014) showed that production of lactic and acetic acids contributes towards the antibacterial activity of Lactobacillus. Furthermore, Lactobacillus mediates antibacterial activity by producing, bacteriocin, H2O2, CO2 and also acts as an epithelial barrier to prevent pathogen adhesions to the mucosa by maintaining tight junctions and inducing mucin secretion (Chen et al., 2019). Lactobacillus modulates the immune system by stimulating the production of cytokines, immune cells, and antibodies resulting in signaling pathways (Hemarajata and Versalovic, 2013).

Probiotics have been applied widely in fresh milk and fermented food products including dairy products, pickles, olives, and kefir (Rezac et al., 2018). Lactobacillus is the commonly used probiotic in the food industry due to its tremendous health benefits to the consumer (Dincer, 2019).

Significance of probiotic cheddar cheese and Lactobacillus

Probiotic cheddar cheese is a processed product with viable probiotic bacteria in an appropriate matrix with adequate concentration (Stefanovic et al., 2019). Cheddar cheese is offered as a good probiotic carrier due to its physical and chemical properties (high pH, high-fat content, dense matrix, high buffering capacity, harder consistency) compared to other dairy products (Ganesan et al., 2015). These characteristics stipulate the survival of probiotics during cheese production as well as during passage through the gastrointestinal tract facilitating a more favorable environment for probiotics (Castro et al., 2015). Makelainen et al., (2010) indicated that probiotic cheese which contains Lactobacillus able to modulate intestinal microbiota, enhance the phagocytosis process, and stimulate innate immunity causing health benefits to the consumer.

Nanoparticle synthesis, characterization, and applications

As a consequence of massive development in nanotechnology, the utilization of nanomaterials has been raised among the global population. The innovative applications of nanotechnology expanded in different fields such as biotechnology, biomedical science, pharmaceutical, food, and agriculture (Mobasser and Firoozi, 2016). Different approaches have been developed to synthesize nanoparticles including physical, chemical, and biological methods (Satyanarayana, 2018). Though physical and chemical methods being used for decades, currently researchers are moving towards biological methods owing to its favorable advantages (Mandava, 2017). Comparatively, Biological

methods are more ecofriendly, simple, non-hazardous, cost-effective, and high yieldable (Ajmal et al., 2019).

Biosynthesis of nanoparticles is considered eco-friendlier due to the minimal generation of waste with subdued toxic effects on the environment. Furthermore, it is reported that biosynthesis is more beneficial over other methods since biological bases facilitate nanoparticles to be biocompatible with living beings. Therefore, it reduces the risk of medical applications (Das et al., 2017). The antibacterial property of nanoparticles is one of the fundamental medical applications. Gudikandula and Maringanti (2016) revealed that biosynthesized nanoparticles have more effect on antibacterial activity compared to chemical synthesis.

Lactobacillus mediated silver nanoparticles synthesis and antibacterial activity

Among metallic nanoparticles, AgNPs are one of the fascinating nanomaterials used in the medical industry due to their functional properties (Zhang et al., 2016). Moreover, Phull and colleagues (2016) indicated that AgNPs had high antioxidant, antibacterial, antifungal, and cytotoxic activities against pathogenic microorganisms. The antibacterial activity of AgNPs mediated by various mechanisms such as damaging to the bacterial cell membrane and interact with biomolecules, inhibit DNA replication and transcription ultimately leads to cell death.

Following research studies demonstrated that Probiotic Lactobacillus are important candidates for the biosynthesis of AgNPs. Thus, they mediate the bio-reduction process in which metal ions in metal salts accept electrons from NADH dependent bacterial enzyme that enhances the formation of metallic nanoparticle (Sani, Aminu, and Mukhtar, 2017) and also Lactobacillus secrete nitrate reductase enzyme which is responsible for the reduction of Ag+ to AgNPs (Rajesh, Dharanishanthi and Kanna, 2014). Garmasheva et al., (2016) demonstrated that AgNPs synthesized via Lactobacillus strains have greater antibacterial activity against pathogenic bacteria and Lactobacillus afforded ameliorative function to obtain a high quantity of AgNPs.

Significance of the project

Consumers are more concerned about probiotic foods due to its health benefits. Therefore, it is required to identify probiotics in food products to protect consumer's freedom of choice and to maintain the quality of the food. Research studies have indicated biosynthesized AgNPs using Lactobacillus have high antagonistic effects against pathogenic bacteria. Therefore, this study aims to identify Lactobacillus in commercially available cheddar cheese samples and evaluate the antibacterial activity of Lactobacillus cell-free suspension and AgNPs synthesized by isolated Lactobacillus.

METHODOLOGY

Sample collection and preparation

Five cheddar cheese from five different brands were purchased from the local market. These samples were labeled as S1 to S5. 5g of each sample was homogenized. All the techniques were carried out under aseptic conditions.

Culturing and isolation of Lactobacillus on MRS agar

The homogenized sample was cultured on MRS agar using the quadrant streaking method and was incubated at 37o C for 48 hours.

Identification of Lactobacillus by biochemical assays

Identification of Lactobacillus by gram staining

Bacterial smear was prepared by mixing a colony taken from the sample with a drop of water on a glass slide and was heat fixed. The slide was flooded with crystal violet for one minute and Gram's iodine was added. Then, the decolorizer was added and left for 15 seconds. Then the slide was flooded with safranin. Every staining step was followed by rinsing with distilled water. The slide was air-dried and smear was observed under microscope at 100X magnification.

Identification of Lactobacillus by Catalase test

A drop of hydrogen peroxide (H2O2) was mixed with a small amount of bacteria on a glass slide. It was checked for the formation of air-bubbles.

Identification of Lactobacillus by Acid-fast staining

Bacterial smear was prepared on a glass slide and was heat fixed. Carbol fuchsin was added and the heat was provided by a candle. Smear was flooded with 20% H2SO4 till the red smear change into yellow. 90% of alcohol was added and washed after 2 minutes. Smear was stained with methylene blue for 1 – 2 minutes. Every staining step was followed by rinsing with distilled water. The slide was air-dried. Smear was observed under microscope at 100X magnification.

Identification of Lactobacillus by Endospore staining

Bacterial smear was prepared on a glass slide and heat-fixed. A piece of filter paper was placed over the smear and stained with a drop of malachite green. The slides were placed staining rack that was kept over water bath. After 5 minutes, the filter paper was removed. Slides were allowed to cool and rinsed with distilled water. Smear was stained with safranin for 2 minutes and air-dried. Smear was observed under the microscope under microscope at 100X magnification.

Subculture of identified Lactobacillus

The identified Lactobacillus was subcultured in MRS broth and incubated at 370C for 48 hours.

Synthesis of AgNPs by Lactobacillus

Subcultures incubated for 24 hours at 370C were centrifuged at 4000 rpm for 5 minutes. The bacterial pellet was dissolved in 900 µL of autoclaved distilled water. 9mL from the 1mM silver nitrate (AgNO3) was added to prepared 1mL of the bacterial suspension. The solution was exposed to sunlight for 24 hours (Modified from Garmasheva et al., 2016).

Quantification of synthesized AgNPs.

Synthesized AgNPs were quantified by spectrophotometer at a range of wavelengths of 330 - 610 nm.

Scanning electron microscopy analysis of synthesized AgNPs

Synthesized AgNPs were analyzed under SEM.

Determination of antibacterial activity against Staphylococcus aureus and Escherichia coli by well diffusion method

An inoculum of each bacterial culture to be tested was spread on respective Muller Hinton's Agar (MHA). Subsequently, four wells were punched in the agar medium and filled with 50µL of autoclaved distilled water (negative control), gentamycin (positive control), Lactobacillus cell-free suspension, and AgNPs for respective well. Triplicates were performed for each sample. Plates were incubated for 24 hours at 370C and the diameter of the inhibition zones was

measured (Modified from Garmasheva et al., 2016).

DATA ANALYSIS

The inhibition zones were compared using SPSS software through one-way ANOVA (P-value<0.05).

RESULTS

Isolation of Lactobacillus in MRS agar

Morphological characteristics of the isolated bacterial cultures from cheddar cheese samples, after 24 hours incubation are shown in Figure 1.

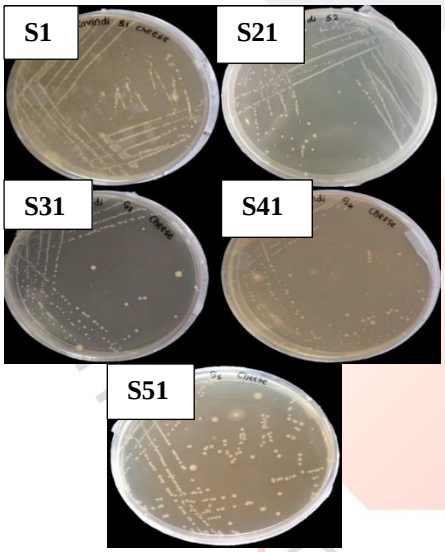


Figure 1. Colony morphology of isolated bacteria culture samples (Sample 1-5).

Creamy white colour, small and large, circular, mucoid, translucent colonies with entire margins were observed for all five samples.

Identification of Lactobacillus by performing biochemical assays

Gram staining

Gram staining of selected colonies, cultured in MRS agar shown in Figure 2.

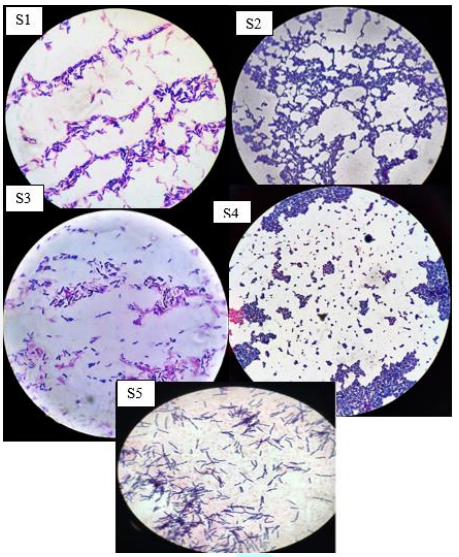


Figure 2. Microscopic view of gram staining for suspected colonies from isolated bacterial cultures (S1-S5) under 100X magnification.

All five samples (Samples 1-5) were appeared in purple, revealing the presence of gram-positive bacteria. Samples 1,2,3 and 5: rod-shaped bacilli, Sample 4: circular clusters (cocci) were observed.

Catalase test

Catalase test performed on previously used colonies for the gram staining (Figure 3).

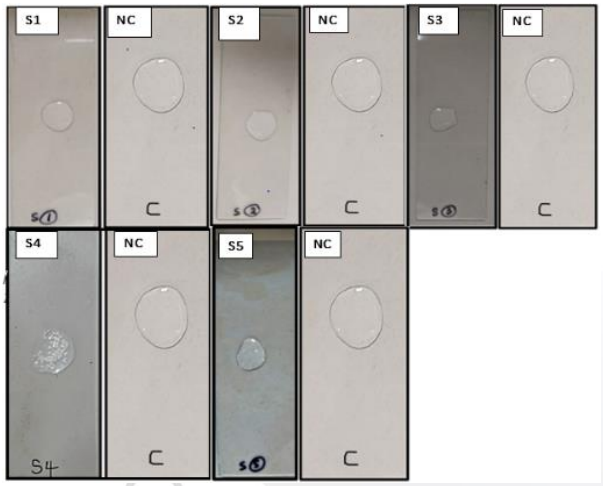


Figure 3. Results of the catalase test for the previously used colonies from each sample. NC: negative control.

Negative results (no air bubble formation) were observed for samples 1,2,3 and 5 representing the presence of catalase-negative bacteria. The positive result (air bubble formation) was observed for sample 4 indicating catalase-positive bacteria.

Acid-fast staining

Figure 4 illustrates the acid-fast staining images

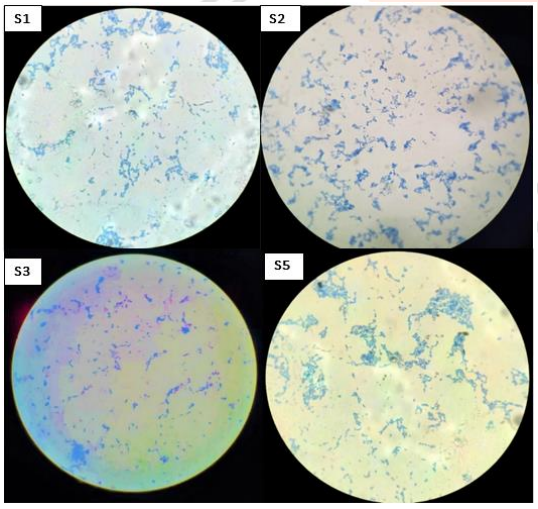


Figure 4. Acid-fast staining image under 100X magnification

All four samples (Sample 1,2,3 and 5) appeared in blue, indicating acid-fast negative bacteria.

Endospore staining

Endospore staining was performed on previously used colonies (Figure 5).

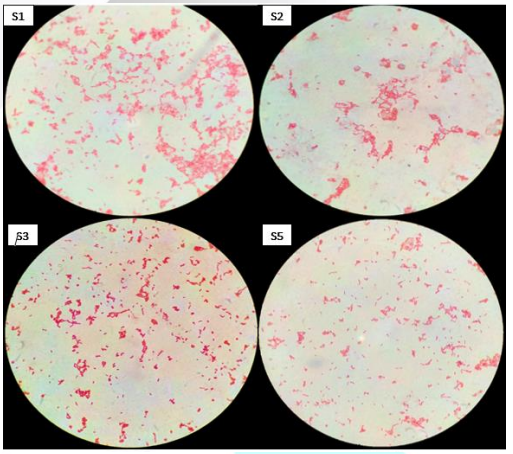


Figure 5. Endospore staining images of Lactobacillus.

All four samples (Samples 1,2,3, and 5) were observed in reddish-pink colour representing non-spore-forming vegetative bacteria.

According to the results obtained from the above biochemical assays, the tested bacteria may be Lactobacillus.

Subculture of colonies to MRS broth

Subcultured MRS broth incubated for 48 hours at 37° C (Figure 6).

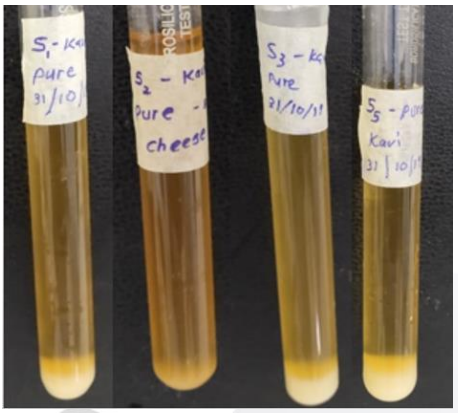


Figure 6. Subculture image of Samples 1,2,3 and 5

Precipitation was observed for all samples (Samples 1,2,3 and 5).

Identification of Lactobacillus by SEM

Figure 7 indicates SEM images of Lactobacillus for samples 2 and 3.

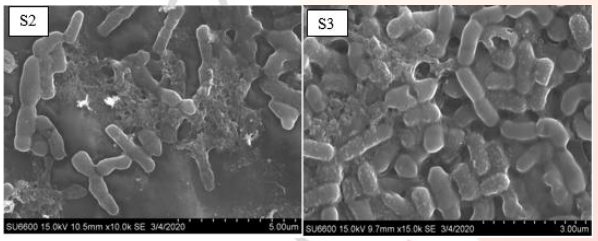


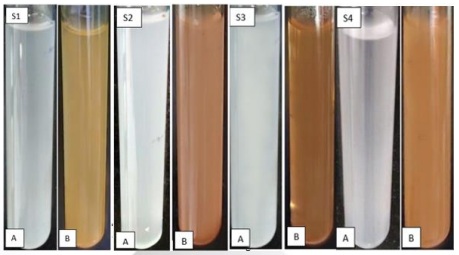
Figure 7. SEM image of Lactobacillus for Samples 2 and 3. Sample 2: 10.0k SE magnification and 5 µm scale. Sample 3: 15.0k SE magnification and 3 µm scale.

Sample 2 indicated rods with both short and long forms while Sample 3 indicated Regular short rods in fissured surfaces.

Synthesis of AgNPs via Lactobacillus

Visible observations of nanoparticles synthesis

The formation of AgNPs was preliminarily observed by the colour change of the solution (Figure 8).



The colour changed from milky white colour to dark brown indicating the formation of AgNPs.

Absorbance curves for synthesized silver nanoparticles.

Figure 9 shows the absorbance graphs of AgNPs for all four samples

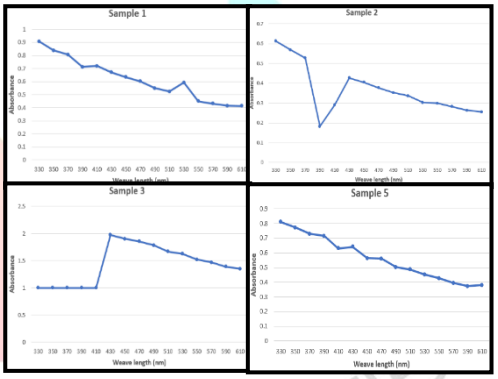


Figure 9. The UV visible absorbance spectra (330-610)nm of AgNPs synthesized using Lactobacillus.

The absorption peaks were obtained in the visible range at a range of 410-440 nm indicating the absorption peak range of AgNPs.

The SEM analysis for detection of silver nanoparticles

Figure 10 shows SEM images of synthesized AgNPs.

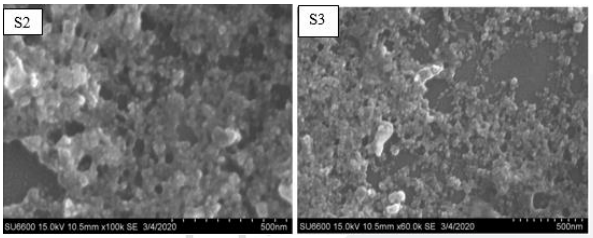
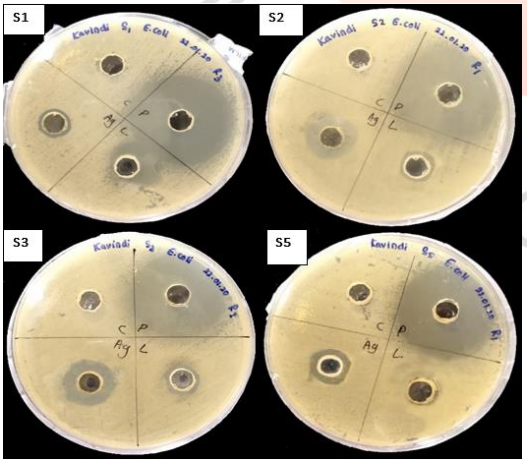


Figure 10. SEM images of synthesized AgNPs at a magnification of 100k SE (S2) and 60.0k SE (S3).

The SEM analysis indicated most of the synthesized AgNPs were well dispersed, spherical shaped with 50- 100 nm in size.

Determination of the antibacterial activity of synthesized AgNPs and isolated Lactobacillus cell-free suspension by well diffusion method

Agar well diffusion plates of Escherichia coli (Figure 11).



S1 indicated a synergistic effect. No inhibition zones were observed for negative control. Only samples 2 and 3 showed inhibition zones for cell-free suspension. All four samples showed an inhibition zone for AgNPs and positive control.

Table 1. Mean of inhibition zones of Escherichia coli

Sample	Positive control (mm)	AgNPs (mm)	Cell-free suspension (mm)	Negative control (mm)
S1	33.00 ± 6.24	11.00 ± 2.64	0.00	-
S2	32.00 ± 4.58	17.00 ± 3.60	10.00 ±1.73	-
S3	34.00 ± 4.35	20.00 ± 1.00	10.00 ± 1.00	-
S5	32.00 ± 2.64	19.00 ± 3.60	0.00	-

Comparatively, AgNPs showed larger inhibition zones for Escherichia coli than cell-free suspension for all four samples.

Agar well diffusion plates of Staphylococcus aureus (Figure 12).

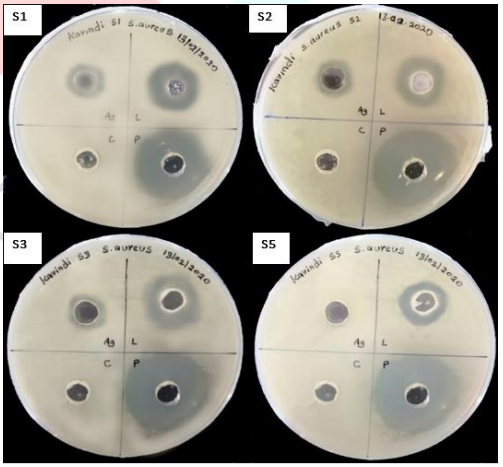


Figure 12. Antibacterial effect of cell-free suspension and synthesized AgNPs on the Staphylococcus aureus. C: autoclaved distilled water, P: gentamycin, Ag: synthesized silver nanoparticles, L: Lactobacillus cell-free suspension

All the samples were indicated inhibition zones for positive control, AgNPs, and cell free-suspension except sample 5 for AgNPs. Inhibition zones were not observed for negative control.

Table 2. Mean values of inhibition zone for Staphylococcus aureus

Sample	Positive control (mm)	AgNPs (mm)	Cell-free suspension (mm)	Negative control (mm)
S1	30.00 ± 6.24	12.00 ± 2.64	21.00 ± 4.00	-
S2	33.00 ± 3.00	10.00 ± 1.73	18.00 ± 1.00	-
S3	27.00 ± 4.58	12.00 ± 1.00	19.00 ± 2.64	-
S5	31.00 ± 2.64	0.00	17.00 ± 1.00	-

Compared to AgNPs, cell-free suspension indicated higher inhibition zones against Staphylococcus aureus for all four samples.

Statistical analysis using one – way ANOVA

ANOVA results for Escherichia coli. (Figure 13)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	216	1	216	12.1121	0.02535	7.70865
Within Groups	71.3333	4	17.8333			
Total	287.333	5				

Figure 13. ANOVA results of the antibacterial activity of AgNPs and Lactobacillus against Escherichia coli. (Significance level = 0.05).

P-value < 0.05, indicating a significant difference between cell-free suspension and AgNPs against Escherichia coli.

ANOVA results for Staphylococcus aureus (Figure 14)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	170.667	1	170.667	8.06299	0.04689	7.708647422
Within Groups	84.6667	4	21.1667			
Total	255.333	5				

Figure 14. ANOVA results of antibacterial activity of AgNPs and Lactobacillus against Staphylococcus aureus. (Significance level = 0.05).

P-value <0.05 indicating a significant difference between cell-free suspension and AgNPs against Staphylococcus aureus.

ANOVA results of cell-free suspension antibacterial activity against Staphylococcus aureus and Escherichia coli (Figure 15)

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	192.667	1	192.667	11.2233	0.02857	7.70865
Within Groups	68.6667	4	17.1667			
Total	261.333	5				

Figure 15. Statistical analysis of cell-free suspension antibacterial activity against Staphylococcus aureus and Escherichia coli (Significance level = 0.05).

P-value <0.05 indicating a significant difference, in the inhibitory effect of cell-free suspension against Staphylococcus aureus than Escherichia coli.

ANOVA results of AgNPs antibacterial activity against Staphylococcus aureus and Escherichia coli (Figure 16).

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	192.667	1	192.667	8.82443	0.04112	7.70865
Within Groups	87.3333	4	21.8333			
Total	280	5				

Figure 16. Statistical analysis of AgNPs antibacterial activity against Staphylococcus aureus and Escherichia coli (Significance level = 0.05).

P-value < 0.05 indicating a significant difference, in the inhibitory effect of AgNPs against Escherichia.coli than Staphylococcus aureus.

DISCUSSION

Currently, probiotic foods are the most frequently used functional foods due to the potential health benefits beyond the inherent basic nutritional effects. Dairy products are a good source of probiotic foods and Lactobacillus species are prominent microorganisms containing probiotic properties. Research studies have indicated that biosynthesized AgNPs using Lactobacillus have high antagonistic effects against pathogenic bacteria and this antibacterial activity of AgNPs significantly applies to treat infectious diseases (Matei et al., 2020). Therefore, this study focused to identify Lactobacillus in cheddar cheese and to evaluate the antibacterial activity of AgNPs synthesized by Lactobacillus.

Colony morphology and phenotypic observation indicated creamy white colour, circular, mucoid, translucent colonies with entire margins (Figure 1). A study carried out by Padmavathi et al., (2018) demonstrated similar results for Lactobacillus colony morphology isolated from dairy products. According to the results of gram staining, four samples (Samples 1, 2, 3, and 5) were obtained as gram-positive bacillus in purple, long and short rods that might be Lactobacillus (Figure 2). These findings were consistent with the results of Sulmiyati et al., (2018). Gram-positive bacteria contain a thick peptidoglycan layer that retains crystal violet-iodine complex. Therefore gram-positive bacteria appear in purple (Toole, 2016).

Samples obtained as gram-positive bacilli were negative for the catalase test due to the absence of air bubble formation (Figure 3) which aligned with the study performed by Abiona and Adegoke, (2017) showed Lactobacillus as a catalase-negative bacteria. Nevertheless, sample 4 was indicated gram-positive cocci in clusters or singles (Figure 2). Air bubbles were observed in the catalase test, indicating a catalase-positive bacteria (Figure 3) suggesting that Lactobacillus is not present in sample 4.

Among the five samples, only gram-positive, rod-shaped catalase-negative bacteria were selected for further investigation.

Results of the acid-fast staining indicated, blue colour acid-fast negative bacteria (Figure 4). Similary, Goldstein, Tyrrell and Citron (2015) reported Lactobacillus as a non-acid fast bacteria. Acid-fast negative bacteria lack lipoidal mycolic acid in the cell wall. Once it stained with methylene blue, appeared in blue (Miller, Harrington, and Procop, 2015).

Endospore staining resulted in reddish-pink indicating vegetative (non-spore forming) bacteria (Figure 5). Similarly, Mulaw et al., (2019), also have indicated the microscopic view of Lactobacillus without endospores. Vegetative bacteria do not retain malachite green due to lack

of spores. When non-vegetative cells stain with safranin, they appeared in pink (Oktari et al., 2017).

The presence of Lactobacillus was further confirmed by SEM for samples 2 and 3 (Figure 7). Regular rods with short and long forms were observed for isolated Lactobacillus which similar agreement with the results of Kang et al., (2019).

The colour of synthesized AgNPs was changed from white to brown after exposure to sunlight (Figure 8) where this result was compatible with the results obtained by, Sani, Aminu, and Mukhtar, (2017). Kumar et al., (2016) reported that colour change occurs due to the excitation of surface plasmon resonance vibrations in AgNPs with the reduction of Ag+ to Ag. Furthermore, Lactobacillus act as a reducing and capping agent. (Dakhil, 2017).

According to Figure 9, the absorbance spectrum of AgNPs showed multiple peaks at different absorbance values. Dhoondia and Chakraborty (2012), reported that the absorbance of AgNPs was maximum at 430nm. Among these multiple peaks, a peak was obtained at 430nm in Samples 2, 3, and 5 indicating the presence of AgNPs. Sample 1 obtained a peak at 410nm (Figure 9) which aligned with the results of Dakhil, (2017). However, Singh et al., (2017) have obtained the absorption spectrum of AgNPs with a wide range of peaks, which lies between the 400-500nm range. Peak located at a range of 400nm indicated that particles are well distributed without much aggregation (Jaffat, Aldujaili, and Hassan, 2010). This fact has been proven by SEM analysis of synthesized AgNPs (Figure 10) where it indicated AgNPs with spherical-shaped and well-dispersed characteristics and 50-100nm in size. Similar characteristics were obtained by Femi-Adepoju et al., (2019) from biosynthesized AgNPs.

The antibacterial activity of synthesized AgNPs and Lactobacillus cell-free suspension against Staphylococcus aureus and Escherichia coli was evaluated by the well diffusion method. The cell-free suspension contains metabolites produced by Lactobacillus and some of these metabolites are responsible for the antibacterial effect. Figure 11 indicated clear zones around AgNPs and cell-free suspension, suggesting that AgNPs and cell-free suspension able to inhibit the growth of Escherichia coli. Maruthai et al., (2017) confirmed that AgNPs capable of inhibiting Escherichia coli. The inhibitory effect of Lactobacillus metabolites against Escherichia coli was not observed for samples 1 and 5 (Figure 11). This could be due to the presence of different species or strains of Lactobacillus in four samples such as Lactobacillus plantarum and Lactobacillus pentosus which are unable to inhibit Escherichia coli (Ren et al., 2018). Sample 1 indicated a synergistic effect between positive control and Lactobacillus cell-free suspension.

According to Table 1, AgNPs showed higher zones of inhibition (antibacterial activity) against Escherichia coli than cell-free suspension for all four samples and one-way ANOVA analysis (Figure 13) has further confirmed the significant difference in antibacterial activity (p-value <0.05) of AgNPs and Lactobacillus metabolites against Escherichia coli.

As shown in Figure 12 clear zones around cell-free suspension and AgNPs have observed in Staphylococcus aureus, suggesting that Lactobacillus metabolites and AgNPs inhibit the growth of Staphylococcus aureus. Khalil, Allam, and El-Mahallawy (2019) showed that Lactobacillus contains antagonistic metabolites that inhibit the growth of Staphylococcus aureus. Qais et al., (2019) confirmed that biosynthesized AgNPs has an inhibitory effect against Staphylococcus aureus.

In line with diameter values (Table 2), cell-free suspension indicated higher zones of inhibition (antibacterial effect) against Staphylococcus aureus than AgNPs for all four samples, and one-way ANOVA analysis (Figure 14) has further confirmed a significant difference in antibacterial activity (p-value <0.05) of AgNPs and Lactobacillus metabolites against Staphylococcus aureus.

In this study, the antibacterial effect of Lactobacillus metabolites against Staphylococcus aureus was greater than that on Escherichia coli (Figure 11 and Figure 12). The one-way ANOVA (Figure 15) further confirmed that there is a significant difference between the antibacterial activity of Lactobacillus metabolites against Escherichia coli and Staphylococcus aureus (p-value <0.05). Tebyanian et al., (2017) and Zhu et al., (2018) demonstrated that there is a significant difference in antibacterial activity of Lactobacillus against Escherichia coli and Staphylococcus aureus due to the presence of lipoteichoic acids in gram-positive bacteria which interact with bacteriocin produced by Lactobacillus. Less interaction occurs between metabolites of Lactobacillus and Escherichia coli due to the absence of lipoteichoic acids (Peh, Pyar, Liong and 2013). Furthermore, Ayantola and Oladunmoye (2016) reported that the effectiveness of the antibacterial activity of Lactobacillus depends on the amount of inhibitory compounds production (e.g. lactic acid, bacteriocins, H2O2). With reference to Khalil, Fadihl, and Ali (2017), Lactobacillus isolated from yogurt was showed a bacteriostatic effect on Escherichia coli and a bacteriocidal effect on Staphylococcus aureus.

The AgNPs showed larger inhibition zones against Escherichia coli than Staphylococcus aureus in all four samples. The maximum inhibition zone in Escherichia coli was 20mm and 10mm was Staphylococcus aureus (Table 1 and Table 2). These results were further confirmed by the ANOVA table (Figure 16), indicating a significant difference between the antibacterial activity of AgNPs against Escherichia coli and Staphylococcus aureus (P-value <0.05). Al-Sharqi et al., (2019) confirmed the significant (P<0.05) difference in antibacterial activity of AgNPs against Escherichia coli and Staphylococcus aureus by using one-way ANOVA analysis.

According to Soo-Hwan et al., (2011), AgNPs treated Escherichia coli showed more protein leakage, rapid inactivation of lactate dehydrogenase, high bactericidal activities in growth curves than Staphylococcus aureus.

In addition, Loo et al., (2018), reported that AgNPs has more antibacterial effect on gram-negative bacteria such as Escherichia coli. Qasim et al., (2018) and Al-Sharqi et al., (2019), suggested that it might be due to structural variations in cell walls as Gram-negative bacteria contain a thin, negatively charged lipopolysaccharide layer which is more susceptible to attract with positive charges in AgNPs facilitating a greater antibacterial activity. Gram-positive bacteria are less vulnerable to adhesion of Ag ions and antibacterial activity due to the highly cross-linked rigid peptidoglycan layer. AgNPs attach to the negatively charged cell wall, deteriorate cell membrane, interfering with cellular functions including permeability, electron transport, osmoregulation, and respiration. AgNPs interact with DNA, the thiol group of L-cysteine protein, and other cell components causing enzymatic dysfunction. AgNPs induce oxidative stress, subsequently damaging proteins and DNA (Almalah, Alzahrani and Abdelkader, 2019).

Mirzajani, et al., (2011) evaluated, inhibition mechanisms of AgNPs against Staphylococcus aureus by damaging cell wall and accumulate in the cell membrane

of Staphylococcus aureus, resulting in the release of cellular components into the surrounding environment. The high concentration of AgNPs results in the release of muramic acid (MA) into the medium, which could be contributed to cell wall distraction and cell death. Furthermore, Li et al., (2010) showed that AgNPs are able to reduce the enzymatic activity of respiratory chain dehydrogenase and change the expression abundance of some proteins present in the Staphylococcus aureus.

CONCLUSION

In conclusion, Lactobacillus was identified in four cheddar cheese samples and this study demonstrated that Lactobacillus isolated from these samples were able to synthesis AgNPs. Synthesized AgNPs and Lactobacillus cell-free suspension indicated promising antibacterial activity against Escherichia coli and Staphylococcus aureus that causes infectious conditions. Therefore, the antibacterial property of AgNPs can be effectively applied to treat multidrug-resistant pathogenic microorganisms. Furthermore, Knowledge gained from this analysis can be effectively used to treat certain diseases caused by drug-resistant pathogens instead of using antibiotics and other medications.

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