Controllable Biogenic Synthesis of Intracellular Silver/Silver Chloride Nanoparticles by *Meyerozyma guilliermondii* KX008616

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Intracellular synthesis of silver/silver chloride nanoparticles (Ag/AgCl-NPs) using *Meyerozyma guilliermondii* KX008616 is reported under aerobic and anaerobic conditions for the first time. The biogenic synthesis of Ag-NP types has been proposed as an easy and cost-effective alternative for various biomedical applications. The interaction of nanoparticles with ethanol production was mentioned. The purified biogenic Ag/AgCl-nanoparticles were characterized by different spectroscopic and microscopic approaches. The purified nanoparticles exhibited a surface plasmon resonance band at 419 and 415 nm, confirming the formation of Ag/AgCl-NPs under aerobic and anaerobic conditions, respectively. The planes of the cubic crystalline phase of the Ag/AgCl-NPs were confirmed by X-ray diffraction. Fourier-transform infrared spectra showed the interactions between the yeast cell constituents and silver ions to form the biogenic Ag/AgCl-NPs. The intracellular Ag/AgCl-NPs synthesized under aerobic condition were homogenous and spherical in shape, with an approximate particle size of 2.5–30 nm as denoted by the transmission electron microscopy (TEM). The reaction mixture was optimized by varying reaction parameters, including temperature and pH. Analysis of ultrathin sections of yeast cells by TEM indicated that the biogenic nanoparticles were formed as clusters, known as nanoaggregates, in the cytoplasm or in the inner and outer regions of the cell wall. The study recommends using the biomass of yeast that is used in industrial or fermentation purposes to produce Ag/AgCl-NPs as associated by-products to maximize benefit and to reduce the production cost.

Keywords: Ag/AgCl-NPs, ethanol, fermentation, intracellular synthesis, *Meyerozyma guilliermondii*, X-ray, transmission electron microscope

Introduction

Nanotechnology is a multidisciplinary science that deals with very small particles ranging from 1 to 100 nm and has been utilized in many applications such as medicine, pharmacy, industry, and agriculture [1, 2]. Nanoparticles of some metals like Au, Ag, Pt, and Cu have attracted more attention because of their biotechnological benefits [3, 4], owing to their unique chemical and physical properties that differ from the metallic particles [5, 6]. Recently, metal

nanoparticles have emerged as an alternative type of antibacterial agent against strains with high resistance to the classical antibiotics [7, 8], antiviral agents [9], anticancer cells and antitumor [10], antifungal [11], antiprotozoal [12], larvicidal [13], anthelmintic [14], and acaricidal [15] agents.

Nanoparticles can be produced either by physical or chemical methods [3]. However, biological production of such important nanoparticles by microorganisms represent a great achievement because it is economic and eco-friendly [16, 17]. Many microorganisms, especially fungi

and bacteria, were approved for their ability to produce different nanoparticles of metals [18, 19]. Fungi were mentioned as excellent candidates for metal nanoparticle synthesis because they contain many enzymes that induce reduction of the metals [20–23].

Current research focuses on the production of nanoparticles by fungi and bacteria; however, production of nanoparticles by yeasts is still restricted to some species. Agnihotri *et al.* [24] revealed that yeast strains that were isolated from extreme environmental niches, such as marine, psychro, thermo, or acidic habitats, showed a high potential to synthesize metal nanoparticles. Apte *et al.* [25] reported that psychrotrophic marine ascomycetous yeast, *Yarrowia lipolytica*, synthesized silver nanoparticles. Mourato *et al.* [26] reported the successful manufacturing of silver nanoparticles by yeast isolated from an extreme acidic mine. Fernández *et al.* [27] approved the production of silver nanoparticles using the culture supernatants of *Cryptococcus laurentii* and *Rhodotorula glutinis*. They mentioned that these yeasts were chosen for their nitrate reductase activity. These findings support the hypothesis that stress conditions could induce different physiological pathways that lead to the synthesis of nanoparticles by such microorganisms. Despite the wealth of knowledge on bioproduction of silver nanoparticle types by yeasts, only one report has examined the ability of different yeast strains to biosynthesize silver nanoparticle types [28]. They provide the first evidence of yeast strains assisting in the synthesis of silver/silver chloride nanoparticles (Ag/AgCl-NPs).

Yeasts as a specific group of microorganisms were approved for their efficiency of bioaccumulation of heavy metals many years ago [29]. Moreover, we assume that ethanol production by yeasts during the fermentation process represents a kind of physiological stress, in which the vegetative growth nearly ceased, and the cell is turned into a biofactory for the production of ethanol. In addition, investigation of new species of yeasts to synthesize metal nanoparticle types could provide new nanoparticles with distinctive characteristics, such as specific shapes and sizes, that make them more applicable in different fields. From this point of view, we assume that inoculation of the fermentation medium with silver nitrate (AgNO_3) could have a good impact on nanoparticle type production by the fermentative yeasts. We identified yeast isolates that biosynthesized Ag/AgCl-NPs under both aerobic and anaerobic conditions. The purified silver nanoparticle types were characterized by a variety of spectroscopy techniques. In addition, we tested different pH values and reaction temperatures, and factors under aerobic and anaerobic

conditions, to expand our information as to what extent these factors can affect the shape and size of biogenic nanoparticles. Furthermore, we tested the susceptibility of the yeast isolate to produce ethanol during the process of biogenic production of nanoparticles.

Materials and Methods

Isolation and Identification of Yeast Strain

The yeast strain used in this study was isolated from naturally fermented wasted rice. The wasted rice sample was obtained from a restaurant before disposal, and was ground using a laboratory blender to obtain the mash with a diameter of particles smaller than 3 mm. Twenty-five grams of the ground sample was poured into a 250-ml Erlenmeyer flask and the volume was completed to 100 ml with sterilized distilled water. The flask was sealed with a fitted cotton plug and incubated in a rotatory shaker (150 rpm) at 25°C for 5 days until natural fermentation occurred and the odor of ethanol was evident. The desired volume (50 µl) was spread on yeast extract–malt extract agar (3 g/l of yeast extract, 3 g/l of malt-extract, 5 g/l of peptone, and 10 g/l of glucose, pH 6.0) and was allowed to grow for 48–72 h at 25°C [30]. The emerged yeast isolates were purified by streaking on the same agar medium at least twice to get a single colony and then they were kept in slants of the same medium at 4°C for further use. The purified yeast isolate was identified by sequencing of the D1/D2 domain of the 26S rDNA region. The extraction of total yeast genomic DNA was performed according to the procedure described by Hesham *et al.* [31]. The D1/D2 domain of the 26S rDNA region was amplified using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') as mentioned by Kurtzman and Robnett [32]. The amplified D1/D2 fragments were purified and sequenced at Macrogen Company (Korea). The obtained sequence of 26S rDNA was aligned with known 26S rDNA sequences in the GenBank database and the percentage homology score was generated to identify the yeast.

Ag/AgCl-NP Synthesis under Aerobic and Anaerobic Conditions

Yeast extract–malt extract broth medium (YMB) was used as a growth medium either for preparing the yeast inoculum or the production of Ag/AgCl-NPs. The inoculum of the yeast isolate was prepared freshly by growing for 48 h in a rotatory shaker incubator (200 rpm) at 25°C. Five milliliters of the freshly prepared inoculum at a cell density of 10^8 cell/ml was injected into a 250-ml conical flask containing 100 ml of the YMB medium at 25°C in a rotatory shaker incubator (200 rpm) for 12 h. Then, the medium was supplemented with 5 ml of AgNO_3 (100 mM). In the case of aerobic incubation, the conical flasks were plugged with fitted sterilized cotton plugs; however, another set of conical flasks were plugged with fitted plastic screw caps to create an anaerobic atmosphere for the fermentation process. The control flasks were not supplemented with AgNO_3 solution. In both experiments, the

growing incubation temperature was adjusted to 25°C and the pH was 7. The incubated flasks were observed for color change, which was the sign for Ag/AgCl-NPs formation. The time at which the color of the medium changed was recorded.

Purification of the Intracellular Synthesized Ag/AgCl-NPs

After formation of the Ag/AgCl-NPs in the culture, which was indicated by a shift of the color of the medium from pale yellow to dark brown, a known volume of the culture was centrifugated at 5,000 rpm for 10 min to precipitate the yeast cell containing the intracellular Ag/AgCl-NPs. The collected yeast cells were washed three times with addition of distilled water and recentrifuged at the same conditions to discard any residue of the medium. The washed yeast cells were dispersed in a volume of distilled water and were sonicated for 10 min (Qsonica Sonicator, Q700) to liberate the nanoparticles into the solution. The intracellular Ag/AgCl-NPs were purified by centrifuging them at 12,000 rpm for 15 min. The supernatant was discarded, and the pellets were dispersed with distilled water and recentrifuged. This process was repeated three times to remove the free entities and unbound biological molecules from the Ag/AgCl-NPs. The obtained nanoparticles were dried at 50–60°C and stored in a brown-glass container for further characterization.

Intracellular Ag/AgCl-NP Characterization

The biogenic silver nanoparticle types were characterized by ultraviolet-visible (UV-VIS) spectroscopy, transmission electron microscopy (TEM), x-ray diffraction (XRD), and Fourier-transform infrared (FT-IR) spectroscopy. The concentration of purified Ag/AgCl-NPs in the solution was measured with atomic absorption spectroscopy (Model 210 VGP; Buck Scientific, USA) equipped with a silver hollow cathode lamp and air-acetylene flame.

Ultraviolet-visible spectroscopy. A strong absorption of electromagnetic waves is exhibited by metal nanoparticles in the visible range owing to surface plasmon resonance (SPR). The spectral analysis was done by using a Jasco V-570 UV/VIS/NIR spectrophotometer in the range 300–800 nm. The stability of stored Ag/AgCl-NPs biologically synthesized by yeast cells was also determined by UV-Vis spectral analysis.

X-ray diffraction. The formation of the crystalline biosynthesized silver nanoparticle types by yeast cells was checked by XRD analysis using a Shimadzu X-ray (Cu α) Diffractometer XRD-6000 ($\lambda = 1.5406 \text{ \AA}$) operated at 30 kV and 25 with a step of 0.02° in the 2θ range of 20° – 80° .

Transmission electron microscope analysis. The size and surface morphology of the synthesized Ag/AgCl-NPs were studied using JEOL TEM (Model 100 CXII) at the Electron Microscope Unit, Assiut University (Egypt). A drop of the purified nanoparticle dispersion (100 $\mu\text{g/ml}$) was placed on a carbon-coated copper grid and dried at room temperature.

Fourier-transform infrared analysis. FT-IR analysis was performed to determine the possible functional groups in the yeast cells responsible for bioreduction of silver ions and formation of

biogenic intracellular Ag/AgCl-NPs. A very small quantity of the sample was mixed with KBr powder to make a pellet, which was subjected to FT-IR analysis in the Thermo Scientific Nicolet 6700 FT-IR spectroscopy device. For attenuation of noise ratio, 32 scans were taken at the range of 450 – $4,000 \text{ cm}^{-1}$ and the resolution was kept at 8 cm^{-1} .

Effect of Incubation Temperatures on Ag/AgCl-NP Formation

The effect of incubation temperatures (25°C, 30°C, and 40°C) on the biogenic Ag/AgCl-NP production was studied by inoculating the YMB medium with 5 ml of the yeast inoculum under aerobic and anaerobic conditions, as mentioned above. The formation of Ag/AgCl-NPs by yeast cells were determined by UV-Vis spectral analysis.

Effect of pH on Ag/AgCl-NP formation

The effect of initial pH values (3.0, 4.0, 5.5, 6.0, and 7.0) on the biogenic Ag/AgCl-NP production was carried out by inoculating the YMB medium (of different pHs) with 5 ml of the yeast inoculum under aerobic and anaerobic conditions, as mentioned above. The pH was adjusted with 1 N HCl or 0.1 N NaOH. The formation and stability of Ag/AgCl-NPs by yeast cells was determined by UV-Vis spectral analysis.

Ultrathin Sections of Yeast Cells

Yeast cell membrane morphology and localization of the Ag/AgCl-NPs were studied using the transmission electron microscope. Yeast cells were harvested from log-phase cultures (grown under aerobic and anaerobic conditions at different pH values). The yeast cells were fixed in 2% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in an ascending acetone series, and embedded in Spurr's resin. Ultrathin sections (60–90 nm) were cut with an ultramicrotome (Reichert Ultracuts). Staining was done by a double-staining technique using uranyl acetate and lead citrate. Ultrathin sections were examined and photographed with the TEM (JEOL, 100 CXII) operating at 200 kV.

Bioethanol Production by *Meyerozyma guilliermondii* KX008616 in the Presence of Different Concentrations of Silver Nitrate

The inoculum of the yeast isolate was prepared by transferring one loopful of 48-h culture grown on a slant of YMPA into a 250-ml Erlenmeyer flask containing 50 ml of sterilized YMP broth. Production of ethanol was conducted at 25°C in 250-ml glass bottles containing 100 ml of sterilized YMP broth, and the pH was adjusted to 5. The fermentation medium was amended with different concentrations of AgNO_3 (0, 2, 4, 6, and 8 mM). Then, 5 ml of the yeast inoculum (10^8 cell/ml) was added to the fermentation medium. The bottles were plugged with fitted plastic screw caps and were incubated in a rotatory shaking incubator (200 rpm) at 25°C for 60 h under anaerobic condition. Three bottles were used for each concentration of AgNO_3 , and 5 ml was taken each 12 h for ethanol estimation. Concentrations of ethanol in the samples were estimated enzymatically using

estimation kits (K620-100) according to the procedures provided by BioVision (USA) [33].

Results and Discussion

A purified yeast isolate was identified using phylogenetic analysis of 18S rRNA gene sequences. The partial 18S rRNA gene sequence of approximately 614 base pairs of *Meyerozyma guilliermondii* KX008616 has a sequence with 100% similarity to *Meyerozyma guilliermondii* MGYN-1 (KJ641911) that is available in the GenBank database.

Laboratory experiments proved the ability of *M. guilliermondii* KX008616 to produce Ag/AgCl-NPs under aerobic and anaerobic conditions in concentrations of 113.576 and 55.237 ppm, respectively. The color of the inoculated medium changed from yellow to brown owing to the SPR phenomenon, indicative of the reduction of silver ions and formation of bio-nanoparticles. The reduction of AgNO₃ could be conducted by constituents of the yeast cells. Examining the yeast cells using the light microscope showed that those growing either aerobically or anaerobically in growth medium amended with AgNO₃ changed into pale brown color compared with the normal clear cells in the control medium (Figs. 1A–1C). This indicates their ability to reduce the silver ions into Ag-NPs or AgCl-NPs. Interestingly, the aggregation of the cells that appeared during fermentation (Fig. 1C) proved the viability of the cells. This finding indicates the ability of the yeast cell to carry out both fermentation and bioreduction of AgNO₃ at the same time. Production of intra- and/or extracellular nanoparticles of metals by some yeasts like *Candida glabrata*, *Schizosaccharomyces pombe*, *Pichia jadinii*, and *Yarrowia lipolytica* was previously mentioned [34]. The findings support our results and confirms the assumption that the type of nanoparticle production (intracellular, extracellular, or on the cell surface) depends on the organism itself. The

synthesis of silver nanoparticles by yeasts was reported as either intra- or extracellular depending upon the species being taken into use [35]. These authors proved the production of silver nanoparticles by *Saccharomyces cerevisiae* after 36 h of incubation with nutrients and subsequent dilution in 30% ethanol. The reduction of the metal by the yeast cells was suggested through two mechanisms. The first mechanism is the participation of a sulfate or nitrate reductase system that is capable of working in the presence of ATP and NADH or anthraquinone [36]. The other mechanism is via activation of a specific polypeptide chain (PC) involving a repeating sequence of general formula (γ-Glu-Cys)*n*Gly with *n* values commonly ranging from 2 to 6. They might bind to the cationic species, immediately forming a cationic-PC complex that is transported into the vacuole, where it is degraded and the nanoparticles are formed [37, 38]. The formation of silver nanoparticles on the cell surface could be due to the electrostatic interaction between the Ag⁺ and the negatively charged carboxylate groups present either on specific enzymes, proteins like cysteine, or even polypeptides present on the cell wall [39, 40].

The UV-Vis absorbance is a good indicator for confirming the formation of Ag/AgCl-NPs by *M. guilliermondii*. As seen from Fig. 2, the purified nanoparticles exhibited an SPR band at 419 nm, confirming the formation of Ag/AgCl-NPs under aerobic condition. The stability of the biogenic silver nanoparticle types was checked after a considerable period of time (up to 64 days), and it was found that the absorbance spectra of the Ag/AgCl-NPs showed the same plasmonic absorption in the visible region with only a slight shift from 419 to 425 nm [41, 42]. As the particles destabilize with time, the original peak (1st day) will decrease in intensity, which may be due to the depletion of stable nanoparticles, and the peak will be broader, which may be due to the formation of aggregates [43–45].

Fig. 1. Light photographs of yeast cells in control medium (A), in medium supplemented with silver nitrate under aerobic condition (B), and in medium supplemented with silver nitrate under anaerobic condition (C). The color shift from pale to dark brown indicates the intracellular production of Ag/AgCl-nanoparticles.

Fig. 2. UV-visible absorbance spectra of biogenic Ag/AgCl-NPs synthesized intracellularly by *Meyerozyma guilliermondii* KX008616 under aerobic condition at different storage times, pH 7, and 25°C.

The formation of the crystalline intracellular Ag/AgCl-NPs biosynthesized by *M. guilliermondii* KX008616 under aerobic and anaerobic conditions was checked by XRD (Figs. 3A and 3B). Under aerobic conditions, the XRD chart exposed the presence of four characteristic peaks at 2θ values of 38°, 44°, 64°, and 78° that were attributed to the (111), (200), (220), and (311) planes of the cubic crystalline phase of the silver nanoparticles, which coexisted with others, and eight peaks at 2θ values of 27°, 32°, 46°, 55°, 57°, 67°, 74°, and 76° indexed as (111), (200), (220), (311), (222), (400), (331), and (420) were attributed to planes of the cubic crystalline phase of silver chloride [28, 46]. However, under anaerobic conditions, most peaks did not clearly appear. This may be due to the changing of the phase to spherical. This interpretation of the Ag/AgCl-NP diffraction profiles is consistent with the standards set by the Joint Committee on Powder Diffraction Standards (JCPDS Card No. 04-0783, to silver nanoparticles, and JCPDS No. 31-1238, to silver chloride nanoparticles). As seen in Figs. 3A and 3B, there were no other peaks, in the sense that the high purity of Ag/AgCl-NPs and the highly crystalline structure was revealed from the strong and narrow diffraction peaks [47].

The size and shape of the intracellular Ag/AgCl-NPs were determined by TEM (Fig. 4). Measurements of biosynthesized nanoparticles diameters (600 Ag/AgCl-NPs) showed that the average diameter was 16.3 ± 9.01 nm under aerobic condition and 34.4 ± 23.39 nm under anaerobic condition. Most of the intracellular nanoparticles

Fig. 3. X-ray diffraction patterns of the biogenic Ag/AgCl-nanoparticles synthesized by *Meyerozyma guilliermondii* KX008616 under (A) aerobic and (B) anaerobic conditions.

synthesized under aerobic condition were homogeneous and spherical in shape (Fig. 4A). It was clear that the biogenic nanoparticles formed under the aerobic condition was in the range of 2.5–30 nm and displayed polydispersity. Moreover, there was diversity in the shapes of the biogenic nanoparticles under the anaerobic condition, of which spherical and polyhedral nanoparticles were found (Fig. 4C).

FT-IR spectral analysis were carried out to identify the possible functional groups in yeast cells responsible for the reduction of silver ions to silver and silver chloride nanoparticles as well as to identify the capping and efficient stabilization agents in the biosynthesized Ag/AgCl-NPs. The FT-IR spectra for both aerobic and anaerobic conditions are demonstrated in Figs. 5A and 5B, respectively. It was recorded that the infrared spectra are similar, but they showed an observed difference of peak intensity at 3,420 and 1,077 cm^{-1} . The broad absorption band that centered

Fig. 4. Representative transmission electron micrographs of biogenic Ag/AgCl-nanoparticles synthesized intracellularly using *Meyerozyma guilliermondii* KX008616 under (A) aerobic and (C) anaerobic conditions, and (B, D) histograms of particle size distributions.

about 3,420 and 3,412 cm^{-1} for the aerobic and anaerobic conditions, respectively, are characteristic of the O-H or N-H stretching vibration modes of carboxylic acid, and phenolic as well as aromatic amino functions [48]. The shift from 3,412 to 3,420 cm^{-1} may be due to inter- and intramolecular interactions of Ag with the -OH group [49]. The weak peaks at 2,919 and 2,840 cm^{-1} for aerobic and at 2,925 and 2,854 cm^{-1} for anaerobic conditions may be assigned to the stretching vibration of aliphatic C-H group [48, 50]. This shift may be attributed to the reduction of Ag^+ to Ag^0 . The weak absorption peak at 2,360 cm^{-1} corresponds to the O-H stretching vibration of phenolic hydroxyls, and the strong band at 1,645 cm^{-1} may be attributed to the

bending vibration modes of aromatic C=C and C=N and to the C=O of (COOH) in both aerobic and anaerobic spectra [51]. The bands at 1,455 and 1,405 cm^{-1} were assigned to the N-H stretch vibration present in amide linkages of the proteins, which has a role in stability/capping of Ag-NPs [52, 53]. The band at 1,241 cm^{-1} corresponds to C-N stretching of amines. The band of 1,077 cm^{-1} may refer to the presence of C-O stretching of alcohols; this band intensity was higher in the anaerobic condition, indicating more quantity of alcohol extracted. Notably, the absorption bands around 500–550 cm^{-1} represent the stretch for Ag-NPs banding with oxygen from the hydroxyl groups. Therefore, the synthesized nanoparticles were surrounded by proteins

Fig. 5. FT-IR spectra of Ag/AgCl-nanoparticles produced by *M. guilliermondii* KX008616 under (A) aerobic and (B) anaerobic conditions.

and metabolites having functional groups [54, 55]. From the FT-IR studies, it could be confirmed that the amino acid and protein characteristic groups have an ability to bind the metal nanoparticles (*i.e.*, capping of silver nanoparticles) to prevent agglomeration and thereby stabilizing the medium. This means that biological molecules can perform double functions to form and stabilize silver nanoparticles in the aqueous medium.

Effect of Incubation Temperature on Biogenic Ag/AgCl-NP Synthesis

Fig. 6 displays the effect of incubation temperature on the rate of production of Ag/AgCl-NPs by *M. guilliermondii* KX008616 under aerobic and anaerobic conditions. It is obvious that the optimum temperature for acceleration and production of biogenic nanoparticles is between 25°C and

30°C. In the case of aerobic conditions, the maximum SPR peak intensity was detected at 30°C (Fig. 6). This revealed that by increasing the reaction temperature, a narrow peak is developed at the lower wavelength region (416 nm at 30°C), which indicates the formation of smaller nanoparticles, whereas at lower temperature, the peaks observed at higher wavelength regions (434 nm at 25°C) clearly indicate the increase in Ag/AgCl-NP size. These findings agree with the fact that when the temperature is increased, the reactants are consumed rapidly by the formation of silver nuclei, preventing secondary reduction on the surface of silver nuclei, leading to the formation of smaller nanoparticles. In contrast, the maximum SPR peak intensity was detected at 25°C in anaerobic medium (Fig. 6). By comparing the maximum SPR peak intensity in Fig. 6, it could be deduced that the production of biogenic nanoparticles in aerobic medium closed to be a fold of those of anaerobic medium, and was compatible with the XRD charts [44, 45, 56].

Effect of pH on Biogenic Ag/AgCl-NP Synthesis

The medium supplemented with AgNO₃ solution changed from pale brown to dark brown as the pH of the reaction mixture increased (Fig. 7). The intensity of the absorbance peaks was pH dependent. The UV-Vis spectra were recorded after the reaction at different pH values (pH 3–7) under aerobic and anaerobic conditions (Fig. 7). Different pH values (pH 5, 6, and 7) resulted in significant Ag/AgCl-NP absorption bands. The maximum Ag/AgCl-NP bioproduction was at pH 7, of which a narrow peak developed, which indicated the formation of highly dispersed bio-nanoparticles under aerobic and anaerobic conditions [57, 58]. Biogenic synthesis of Ag/AgCl-NPs at pH 3.0 under aerobic and anaerobic conditions showed no characteristic peak while the color of the reaction mixture was slightly changed. This change in color is related to the intracellular nanoparticle bioproduction by cell constituents as indicated by the images of the TEM (Figs. 8A and 8E). Many studies suggested that a variety of biomolecules are involved in biological nanoparticle synthesis, and such biomolecules are likely inactivated for the reducing function, like polysaccharides and proteins under extremely acidic conditions (pH 3.0), and work effectively in neutral and slightly alkaline conditions [59, 60]. On the other hand, the noticeable difference in colors obtained over the ranges of pH could be ascribed to a variation in the dissociation constants (pKa) of functional groups on the biomass involved [45, 58, 61, 62]. Therefore, in the present study, pH 7 was recorded for the biogenic synthesis of Ag/AgCl-NPs.

Fig. 6. Direct visualization and UV-vis absorbance spectroscopy analysis of biogenic Ag/AgCl-nanoparticles synthesized by *Meyerozyma guilliermondii* KX008616 under aerobic conditions at various reaction temperatures (25°C, 30°C, and 40°C). (A) and (C) Color change of the reaction mixture (from left to right: control medium without AgNO₃, and medium supplemented with AgNO₃ at 25°C, 30°C, and 40°C under (A) aerobic and (C) anaerobic conditions; (B) and (D) UV-Vis spectra under aerobic and anaerobic conditions, respectively.

The shape, size, and morphology of the biogenic Ag/AgCl-NPs synthesized intracellularly using *M. guilliermondii* KX008616 under aerobic and anaerobic conditions at various pH values (3, 4, 5, 6, and 7) were analyzed by TEM. Figs. 8A–8D of the intracellular Ag/AgCl-NPs synthesized under the aerobic condition at various pH values demonstrate the polydispersity nature of the nanoparticles, with clear uniform shape and size. The biogenic Ag/AgCl-NPs were spherical and ovoid shaped with an approximate particle size of 2.5–30 nm. Thus, *M. guilliermondii* KX008616 is capable of biosynthesizing Ag/AgCl-NPs small in size and more sufficient for bioapplication under the aerobic condition at pH 6 and 7. Moreover, the biogenic Ag/AgCl-NPs synthesized under the anaerobic condition at various

pH values were examined (Figs. 8E–8H). It was clear that the biogenic nanoparticles with a larger size were in the range of 5–80 nm and displayed polydispersity. There were dissimilarities in the shapes of the biogenic nanoparticles based on the images of the TEM, of which spherical, triangular, hexagonal, and polyhedral nanoparticles were found under anaerobic condition. The nanoparticle formation of different shapes and sizes could be due to the presence of more than one reducing agent in yeast cells.

Ultrathin Section of Yeast Cells during the Formation of Biogenic Nanoparticles

TEM study of yeast cells revealed that the biosynthesis of Ag/AgCl-NPs was occurring inside the cell. *M. guilliermondii*

Fig. 7. Direct visualization and UV-Vis absorbance spectroscopy analysis of biogenic Ag/AgCl-nanoparticles synthesized by *Meyerozyma guilliermondii* KX008616 under aerobic conditions at 25°C and various pH values (3, 4, 5, 6, and 7).

(A) and (C) Color change of the reaction mixture (from left to right: control medium without AgNO₃, and medium supplemented with AgNO₃ at pH 3, 4, 6, and 7 under (A) aerobic and (C) anaerobic conditions; (B) and (D) UV-Vis spectra under aerobic and anaerobic conditions, respectively.

KX008616 grown under aerobic and anaerobic conditions and exposed to an aqueous solution of the silver nitrate resulted in a biogenic intracellular activity of the Ag/AgCl-NPs. Fig. 9 shows that at pH 3, yeast cells grown aerobically formed the biogenic nanoparticles inside the cells; in the cytoplasm as clusters. These clusters are known as nanoaggregates [28]. The reduction, in this case, was much slower and the production yield of nanoparticles was little. At pH 5 and 7, intracellular Ag/AgCl-NPs were arranged as nanoaggregates along the cell wall. Figs. 9B–9D show nanoaggregates mostly associated with inner and outer regions of the cell wall with uniform shapes and varied sizes. Additionally, yeast cells were grown aerobically at pH 5 and 7, and besides accumulating silver nanoparticle

types inside the cell, biogenic nanoparticles were secreted outside the cell [63].

In order to investigate the variation in shapes and sizes of intracellular biogenic Ag/AgCl-NPs under the anaerobic condition at various pH values, we examined yeast cells by ultrathin sections with TEM (Fig. 10). At pH 3, yeast cells slightly formed intracellular nanoparticles inside the cells, of which a small population was formed in the cytoplasm away from the cell wall (Figs. 10A and 10B). It was clear that yeast cells grown anaerobically at pH 5 synthesized nanoaggregates in the cytoplasm and in the cell wall (Figs. 10C and 10D). At pH 7, Ag/AgCl-NPs were confirmed in the outer and inner regions of the cell wall (Figs. 10E–10F) and no clear association with the cytoplasm or intracellular

Fig. 8. Transmission electron micrographs of biogenic Ag/AgCl-nanoparticles synthesized intracellularly using *Meyerozyma guilliermondii* KX008616 under (A–D) aerobic and (E–H) anaerobic conditions at 25°C and various pH values.

structures was found. The variation in the shape and size are due to the interaction of different intracellular structures with different crystallographic faces of the Ag/AgClNPs. It was confirmed that yeasts containing nitrate reductase enzyme involved silver nanoparticle production because

Fig. 9. Transmission electron micrographs of yeast cells (*Meyerozyma guilliermondii* KX008616) during the formation of biogenic Ag/AgCl-nanoparticles under aerobic condition at 25°C and various pH values.

(A) Control yeast cells. (B–D) Yeast cells exposed to AgNO₃ at pH 3, 5, and 7.

this enzyme seems to be involved in silver nanoparticle synthesis [64, 65].

Bioethanol Production by *Meyerozyma guilliermondii*

The production of ethanol was assessed during the fermentation period and the results confirmed that after 36 h, the maximum production of ethanol was achieved by *M. guilliermondii* KX008616 in the fermentation medium without AgNO₃. However, a low concentration of AgNO₃ (2 mM) enhanced the productivity of ethanol by this yeast, which reached 4.0 g/l after 48 h of incubation. In contrast, the higher concentrations of AgNO₃ (4–6 mM) had a negative impact on the production of ethanol by the same yeast. The highest concentration of AgNO₃ creased the production of ethanol after 12 h, which indicated the toxic effect of this dose of metal on yeast cells. The results confirm that the yeast *M. guilliermondii* KX008616 is a Ag-resistant strain that could resist the concentration of the metal to 6 mM. However, higher concentrations could be toxic to the cells of this yeast. In accordance with our findings, Kowshik *et al.* [66] reported that the yeast strain MKY3 synthesized silver nanoparticles extracellularly

Fig. 10. Transmission electron micrographs of yeast cells (*Meyerozyma guilliermondii* KX008616) during the formation of biogenic Ag/AgCl-nanoparticles under anaerobic condition at 25°C and various pH values.

(A–F) Yeast cells exposed to AgNO₃ at pH 3, 5, and 7.

during its growth in a medium supplemented with 1 mM silver, and this strain denoted a silver-tolerant yeast. There is no available information about the effect of silver metal on the ethanol production by fermentative yeasts. However, we could assume that the production of ethanol decreased with increase in silver concentration up to 6 mM and was completely inhibited at 8 mM because of the toxic effect of the metal ions on vegetative growth of the cell that could interfere with or suppress the enzyme reactions.

Our results proved for the first time the intracellular synthesis of Ag/AgCl-NPs by *M. guilliermondii* KX008616 under both aerobic and anaerobic conditions. Results showed the variation in size and shape among the Ag/AgCl-NPs that were synthesized either aerobically or

Fig. 11. Production of ethanol by *Meyerozyma guilliermondii* KX008616 in the presence of different concentration of AgNO₃ (2–8 mM) under anaerobic conditions.

Bars represent the standard error.

anaerobically. In aerobic condition, the Ag/AgCl-NPs were homogeneous and spherical in shape with an approximate particle size of 2.5–30 nm. However, under anaerobic conditions, the biogenic nanoparticles were larger in size (5–80 nm) and displayed polydispersity. Our results assume that intracellular, extracellular, or surface production of the nanoparticle by this yeast could be pH-dependent. However, more indepth research is needed to define the exact mechanism that could be involved in this procedure. In addition, production of ethanol by this yeast strain in the presence of a high concentration of silver (6 mM) confirms its resistance to the metal and could be denoted as a silver-tolerant yeast. Furthermore, the ability of this yeast to produce Ag/AgCl-NPs from the culture medium could encourage use of the biomass of yeasts from industrial or fermentation purposes for the production of Ag/AgCl-NPs as associated by-products to maximize benefit and to reduce the production cost.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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