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Research Article **Microbiology**

Evaluating the role of nitrate reductase and the manipulation of the culture conditions on the biogenesis of silver nanoparticles by the wild yeasts

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KEY WORDS

Nitrate reductase, Silver nanoparticles, *Wickerhamomyces onychis*, Culture conditions.

ABSTRACT

This study investigated the correlation between silver nanoparticles (AgNPs) production by yeast isolates, and the activity of nitrate reductase. It also evaluated the influence of different physical and chemical conditions on the growth of *Wickerhamomyces onychis* (*W. onychis*) and AgNPs formation. The NO_2^- concentration of each sample was determined. The result showed that although all the yeasts are capable of synthesizing AgNPs, only eight produced the enzyme nitrate reductase. The relationship between the synthesis of silver nanoparticles and the nitrate reductase enzyme exhibited a statistically insignificant negative association. The development of *W. onychis* and the synthesis of AgNPs were significantly affected by variations in the incubation period, pH, temperature, concentrations of NaCl, and concentrations of various metals. The most effective medium for *W. onychis* growth was YPD medium, while PDB medium was optimal for AgNPs synthesis. The optimal period for the AgNPs production and yeast growth was seven days. The pH level of the media clearly influenced the synthemperature for the formation of AgNPs and the growth of cells was 30°. The optimal conditions for yeast growth and nanoparticles production were determined to be pH 8, 30°C, the absence of sodium chloride, 2 ppm of calcium chloride ($CaCl_2$) and magnesium sulfate ($MgSO_4$), 1 ppm of potassium dihydrogen phosphate (KH_2PO_4), 1.5 ppm of iron chloride ($FeCl_3$), and 1 ppm of cobalt chloride ($CoCl_2$).These results contribute to a better understanding of the potential for quick and inexpensive biosynthesis of metal nanoparticles in the future.

Introduction

Recently, there has been a lot of interest in silver nanoparticles (AgNPs) due to their numerous applications in bimolecular detection, catalysis, antibacterial, antiviral, and anticancer activity. **(Dhaka *et al.*, 2023a; Karunakar *et al.*, 2024; Kumar *et al.*, 2024; Mohamed *et al.*, 2022; Sakthi Devi *et al.*, 2022).** AgNPs have been employed as antibacterial agents in water treatment, food preservation, cosmetics, textile coatings, and other applications for many years **(Dhaka *et al.*, 2023b; Du *et al.*, 2017)**.

Metal nanoparticles have been generally synthesized through different physical and chemical methods. However, these conventional methods have various drawbacks, such as high energy consumption, high cost and the involvement of toxic chemical substances. Using biological resources like fungi, bacteria, and plants to synthesize nanoparticles has emerged as a promising technique for producinion of AgNPs in recent years **(Raj *et al.*, 2022)**. Because of their high secretion of proteins, enzymes, and metabolites, ease of handling during large-scale production, and low cost requirements for manufacturing operations, yeasts and fungi are a more efficient candidate for the biosynthesis of nanoparticles **(Jalal *et al.*, 2018; Kulkarni *et al.*, 2023; Moghaddam *et al.*, 2015; Roychoudhury, 2020; Soliman *et al.*, 2018).**

One mechanism for producing metal nanoparticles involves the reduction caused by the presence of nitrate reductase in biological agents **(Rose *et al.*, 2023)**. Nitrate reductase may be a crucial component of all organisms that produce silver nanoparticles **(Mikhailova, 2020)**.

This enzyme was found to be responsible for the extracellular synthesis of nanoparticles through bioreduction of metal ions by many fungi and yeasts. Studies showed that the presence of nitrate reductase enzyme is essential for the biosynthesis of silver nanoparticles. Therefore, **Fernández *et al.*, (2016)** chose the nitrate reductase-producing yeasts *Candida laurentii* and *Rhodotorula glutinis* for the manufacture of silver nanoparticles using a-NADPH-dependent nitrate reductase.

Cannons *et al.*, (1986) found that the nitrate reductase enzyme is produced by *Candida nitratophila* and that nitrate medium plays a role in increasing the synthesis of enzyme. Amongst the different metal nanoparticles, silver nanoparticles were obtaining important potential in whole world, **Kumar *et al.*, (2007)** established the synthesis of silver nanoparticles employing a-NADPH-

dependent nitrate reductase *in vitro*, where the reduction of silver ions in the presence of nitrate reductase resulted in the creation of stable silver nanoparticles.

The rate of intracellular NPs creation was also influenced, either directly or indirectly, by exposure to different temperature, pH, and substrate concentrations **(Gericke & Pinches, 2006)**. In the present study the relationship of this enzyme with the synthesis of silver nanoparticles and optimization of the medium culture are investigated to achieve the highest yield from AgNPs by *Wickerhamomyces onychis*. The current investigation attempts to evaluate the efficiency of the nitrate reductase enzyme in the AgNPs-producing yeast isolates as well as the impact of various factors on AgNPs generation.

Material and Method

Wild yeasts and fermenting media

Thirty-one wild yeasts, isolated from dry foods and vegetables and noted for their capacity to synthesize silver nanoparticles, were examined to evaluate their production of nitrate reductase and its role in the formation of silver nanoparticles (AgNPs). In our prior research, *Wickerhamomyces onychis* (Y23) (*W. onychis* MSN) (NCBI accession number PP067751) demonstrated the greatest capacity for generating AgNPs. Consequently, it was selected for further experiments. The isolates were initially cultured on a potato dextrose broth medium (PDB) (seed culture), comprising a filtrate derived from boiling 200g of potato, 20g of dextrose, and 1000ml of distilled water. The medium's pH was calibrated to 6.5. The cultivation was carried out at a temperature of 30°C, 120 rpm, 36 hours. Subsequently, 5ml of the seed culture with an optical density of 1.0 at a wavelength of 600nm (OD_{600nm}) were transferred to a fresh potato dextrose (PD) broth media. The cultivation was carried out under the above-described culture conditions for 5 days.

Synthesis and evaluation of AgNPs

The yeast isolates' capacity to synthesize silver nanoparticles (AgNPs) was evaluated in accordance with **Zahran *et al.*, (2013)**. For biomass production, PDB medium was utilized, First, 50ml of sterile broth media in a 250ml Erlenmeyer flask were seeded with 5ml of a 36-hour-old yeast culture (OD_{600nm}= 1.0).

The flasks were incubated for seven days at 150rpm and 28±2°C. Then, the culture media was centrifuge at 4000 rpm for 30 minutes at room temperature. The collected biomass was resuspended in fifty milliliters of sterile deionized water in a 250 milliliter Erlenmeyer flask (OD_{600nm}=2.5) after three washes with sterilized double distilled water. The suspension was then incubated for two

hours at 40°C in a water bath **(Gautério *et al*., 2023).**

The resultant yeast suspension was then combined with a 1mM silver nitrate solution in a tinfoil-covered flask, and the mixture was incubated for 24 hours at 150rpm and 37°C in the dark. After incubating for 24 hours, the Implen NanoPhotometer®, Instrument Type N60, Version NPOS 3.1f build 13220, Serial Number T61180, was used to detect the production of AgNPs in the suspension at absorbance spectra (300-700 nm) **(Yaseen *et al*., 2020).**

Assay of nitrate reductase activity by the yeast isolates based on NO_2^- concentrations obtained from the reduction of NO_3^- to NO_2^-

The enzyme nitrate reductase was evaluated in the yeast culture's supernatant using the approach outlined by Harley **(Harley, 1993)** and Saifuddin *et al*.,(2009). A 5 mL of the yeast culture supernatant was mixed with 5mL of the assay medium, which consisted of 4.6 mL of 0.1 M potassium phosphate buffer (pH of 7.5), 150µl of 1 M KNO_3, and 250µl of 99.9% propanol. The mixture was then agitated (120rpm) at room temperature for one hour. After incubation, 1.25 mL of a solution A (0.3 g of sulphanilamide (molecular weight equals 172.2g/mol) in 100mL of glacial acetic acid), and 1.25 mL of solution B (0.015 g of N-(1-naphthyl) ethylene diamine dihydrochloride (molecular weight equals 259.18g/mol) in 100mL of glacial acetic acid), were added to the assay mixture in order to measure the quantity of nitrites produced. A UV-Vis spectrophotometer was utilized to quantify the generated color at a wavelength of 540nm. To quantify the amount of nitrite generated through enzymatic activity throughout a 60-minute timeframe, a standard curve for nitrite was generated by combining 5ml of the assay medium with 5ml of different nitrite concentrations ranging from 10 to 60 µmol NO_2^-. The solution was stirred at a speed of 120rpm at the ambient temperature for duration of one hour. To quantify the quantity of nitrites generated, 1.25mL of a solution A and 1.25mL of a solution B were introduced into the assay mixture. The generated color was measured at 540nm using a UV-Vis spectrophotometer. The enzyme activity was finally expressed in terms µmol of nitrite $h^{-1}ml^{-1}$ **(McCashin, 2000; Saifuddin *et al*., 2009; Fernández *et al*., 2016; Kim & Seo, 2018; Yaseen *et al*., 2020)**.

Characterization of silver nanoparticles

The synthesized silver nanoparticles were characterized using the following methods:

X-Ray Diffraction (XRD) Analysis

The degree of purity of the silver nanoparticles was determined using powder X-ray diffraction. The biosynthesized silver nanoparticles were

dried in a vacuum drying oven at 45°C. The desiccated powder was utilized for X-ray diffraction (XRD) analysis using an X-ray powder diffractometer (D2 PHASER 2nd Generation, Bruker AXS, Germany) equipped with CuKa1 radiation and a configurable divergence slit. The XRD analysis was conducted with a voltage of 40 kV and an X-ray source current of 30 mA **(Krumm, 1994)**. The Debye Scherrer equation, $D = 0.9\lambda / \beta Cos\theta$, was used to calculate the crystalline size of the nanoparticles, where β represents the full width at half maximum (FWHM) of a peak and λ is the wavelength of the X-rays utilized for diffraction and $Cos\theta$ represents the observed peak angle. The observed peaks were fitted using a Gaussian function using origin 8.5 in order to estimate FWHM.

Transmission electron microscopy (TEM)

The morphology and dimensions of the synthesized nanoparticles (NPs) were examined using transmission electron microscopy (TEM) with a JEM-2100 electron microscope from Japan, operating at an accelerating voltage of 200 kV. A drop of silver nanoparticle solution was applied onto a copper grid that had been coated with carbon, and it was allowed to completely dry for one hour. Microscopic sights have been observed and documented with clarity across various magnifications **(Williams, 1996).**

Effect of chemical and physical conditions on yeast growth and production of AgNPs

The environmental conditions, both physical and chemical, significantly influence the growth of yeast and its metabolic activity, thereby impacting its capacity to produce silver nanoparticles. As a result, an extensive examination was performed on numerous physical and chemical properties to investigate their impact on yeast growth and their capacity to produce silver nanoparticles.

Incubation period

The inoculated PDB media were incubating at 28°C and 120rpm. For seven days. 15ml was systematically harvested at certain intervals to measure the rate of growth and assess their capacity to synthesis AgNPs as described above.

Growth media

Five distinct media - PDB, MYGP (Malt extract yeast glucose peptone), YPD (Yeast peptone dextrose), CD (Czapek Dox), and MCD (Modified Czapek Dox)- were employed to investigate the impact of nutritional conditions on yeast growth and the synthesis of AgNPs by yeasts. The flasks were inoculated and incubated as described above, and then the collected biomass was used for AgNPs synthesis.

pH Parameter

The pH of the PDB media was calibrated to 4, 5, 6, 7, and 8, and then the further

steps were carried out as describe above to evaluate the growth rate and AgNPs production.

Temperature

As previously mentioned, the PDB flasks were inoculated by the seed culture, then incubated at 25°C, 30°C, and 35°C for seven days at 120 rpm. After the growth rate was determined, the cells were gathered to synthesize silver nanoparticles.

Effect of different concentration of NaCl, KH_2PO_4 and $CaCl_2$ on the growth and AgNPs synthesis.

Before autoclaving the PDB media for 20 minutes at 120°C, (0%, 0.5%, 1%, 1.5%, 2%, 2.5%, and 3%) of NaCl and (0, 1, 2, 3) PPM of (KH_2PO_4 and $CaCl_2$) were added separately. The seed culture was added to the aseptic media, and then was incubated for seven days under the previously mentioned conditions. The cells were then collected in order to synthesize silver nanoparticles once the growth rate was measured.

Effect of metal ions ($CoCl_2$, $FeCL_3$ and $MgSO_4$) on the growth and AgNPs synthesis.

Prior to sterilization, separate additions of (0, 0.1, 0.5, 1.0, 1.5) PPM of $CoCl_2$ and $FeCl_3$ and (0, 1, 2, 3) PPM of $MgSO_4$ were added to the PDB media. After inoculating with the seed culture and incubation for seven days, the growth rate was determined, and the cells were gathered to create silver nanoparticles.

Statistical analysis

Triplicate runs of the experiments were conducted. A mean and standard deviation are used to represent data. A Tukey's HSD post hoc test was run after an ANOVA for statistical analysis, and a *P* value of less than 0.05 was deemed significant. The correlation between silver nanoparticles production and nitrate reductase production were carried out using SPSS 27 (https://www.ibm.com/spss).

Result

Assay of nitrate reductase activity by the yeast isolates based on NO_2^- concentrations obtained from the reduction of NO_3^- to NO_2^-

To validate the role of nitrate reductase in the production of silver nanoparticles, thirty-one yeast isolates which are known by their capacity for making silver nanoparticles were used in this study. The AgNPs synthesis was evaluated through color shifting to dark brown color **(Fig. 1a,b)** and detecting the appearance of the peak at 420nm **(Fig. 1c,d)**. While the nitrate reductase was evaluated after incubation of yeast's culture supernatant with assay media for one hour, then 2.5mL of solution A and solution B were added. A pink colored complex (positive result) was produced as a result of the reaction which is measured at 540nm. Each sample's NO_2^- concentration was determined using the formula (y = 0.0609x + 0.0725) getting out

from the standard curve of nitrite NO_2^- (10-60µmol), **Fig. (2a, b)**.

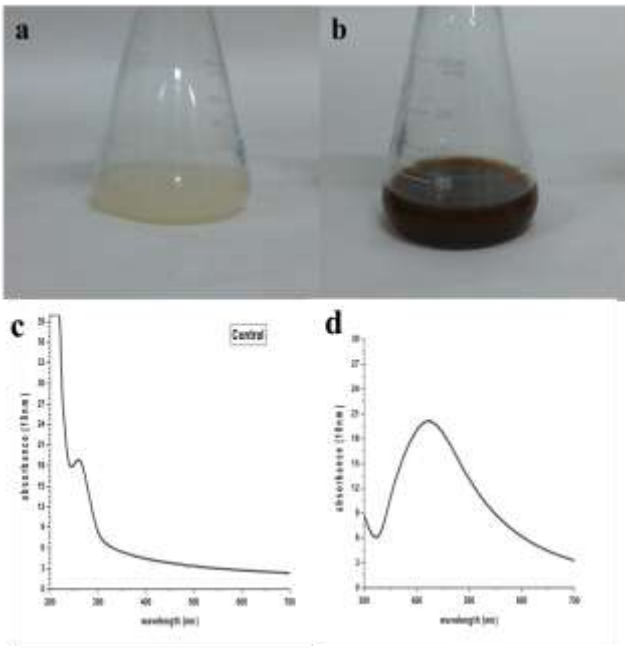

Fig. (1): Detection of AgNPs synthesis by the yeast strains (a) The mixture (collected biomass with 1mM silver nitrate) before incubation (b) The mixture (collected biomass with 1mM silver nitrate) after incubation. (c) UV.vis spectra of the yeasts filtrates without silver nitrate. (d) UV.vis spectra of the yeasts filtrates with $AgNO_3$ indicating the formation of Ag Nanoparticles that is manifested by the appearance of a peak at 420nm.

According to our result presented at **Table (1)** not all the silver nanoparticles producers' strains showed a positive nitrate reductase test. Only eight yeast strains (Y2, Y8, Y10, Y11, Y13, Y24, Y25 and Y29) are found to be able to produce the enzyme nitrate reductase which could catalyze the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). The nitrate reductase producers are classified as a weak and a moderate AgNPs producers except Y24, it yielded the highest amount of nitrate reductase enzyme and classified as a good AgNPs producer. Also, the correlation between the production of AgNPs and nitrate reductase by the yeasts showed a non-significant negative correlation between both variables. This indicates that the nitrate reductase did not play a significant role in the synthesis of silver nanoparticles by yeasts **(Table 2)**.

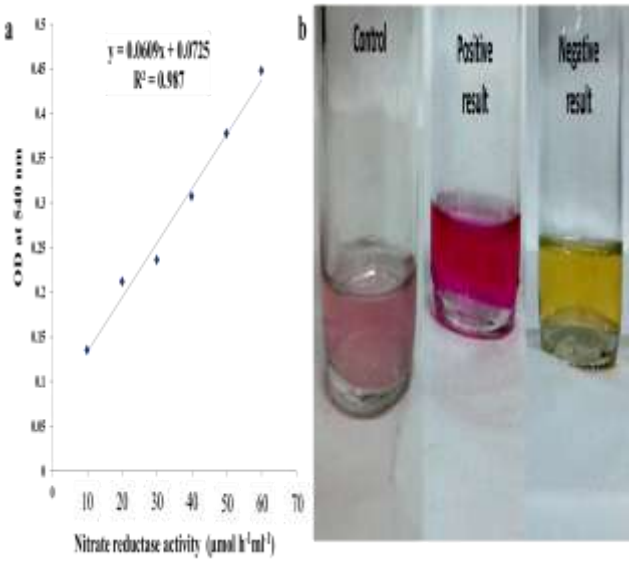

Fig. (2): Estimation of the ability of different silver nanoparticles yeast strains producers on the production of nitrate reductase. (a) Standard curve of nitrite NO_2^- (µmol h-1ml-1) at 540nm; and (b) color shifting to the pink color after reduction of nitrate (NO_3^-) to nitrite (NO_2^-).

Characterization of AgNPs produced by *W. onychis*.

The size and morphology of the biosynthesized AgNPs were detected using the TEM analysis and XRD.

Table (1): NO_2^- concentration of each strain through the equation (y = 0.0609x + 0.0725), where x= NO_2^- conc and y= Od of the sample at 540nm. AgNPs production of each strain which represented as weak producer (+), moderate producer (++) and strong producer (+++).

Strain	Nitrate Reductase Activity (μmol $h^{-1}ml^{-1}$)	AgNPs Production
Y1	ND	++
Y2	$1.17\pm.020^{no}$	+
Y3	ND	+
Y4	ND	++
Y5	ND	++
Y6	ND	++
Y7	ND	+++
Y8	$0.73\pm.007^{mn}$	+
Y9	ND	++
Y10	$2.62\pm.008^{q}$	++
Y11	$0.59\pm.006^{m}$	+
Y12	ND	+
Y13	$1.92\pm.013^{p}$	+
Y14	ND	++
Y15	ND	++
Y16	ND	++
Y17	ND	+
Y18	ND	+
Y19	ND	++
Y20	ND	++
Y21	ND	+++
Y22	ND	++
Y23	ND	+++
Y24	$4.24\pm.019^{s}$	+++
Y25	$1.37\pm.022^{o}$	++
Y26	ND	++
Y27	ND	++
Y28	ND	++
Y29	$3.28\pm.008^{r}$	+
Y30	ND	++
Y31	ND	++
Y32	ND	++
Y33	ND	++
Y34	ND	++

ND, not detected; Mean with different letters is significant (P < 0.05).

Table (2): Correlation between the ability of the studied yeast strains on the production of AgNPs and production of nitrate reductase.

		Nitrate reductase producers
AgNPS producers	Pearson Correlation	-.053
	Sig	.767

According to TEM images, the synthesized nanoparticles exhibited a regular spherical shape with smooth surfaces and the size is ranged between 1.8–11.65nm as shown in **(Fig.3a)**. TEM Selected area electron diffraction (SAED) image shows crystalline property of silver nanoparticles **(Fig.3b)**.

According to **(Fig.3c)**, the XRD pattern of AgNPs showed three main diffraction peaks at 2θ: 37.78°, 44.42°, and 64.53° corresponding to (1 1 1), (2 0 0), and (2 2 0) planes, respectively. The silver nanoparticles formed have the face-centered cubic crystal structure. The average particles size, calculated from the main (1 1 1) diffraction peak width, was about 2.89 nm depends on Deby-Seherrer formula. Through estimating the FWHM, the observed peaks were fitted with a Gaussian function using origin 8.5. The average particles which nearly matches well the obtained particles diameters from TEM analysis **(Fig.3a) (Olobayotan & Akin-Osanaiye, 2019).**

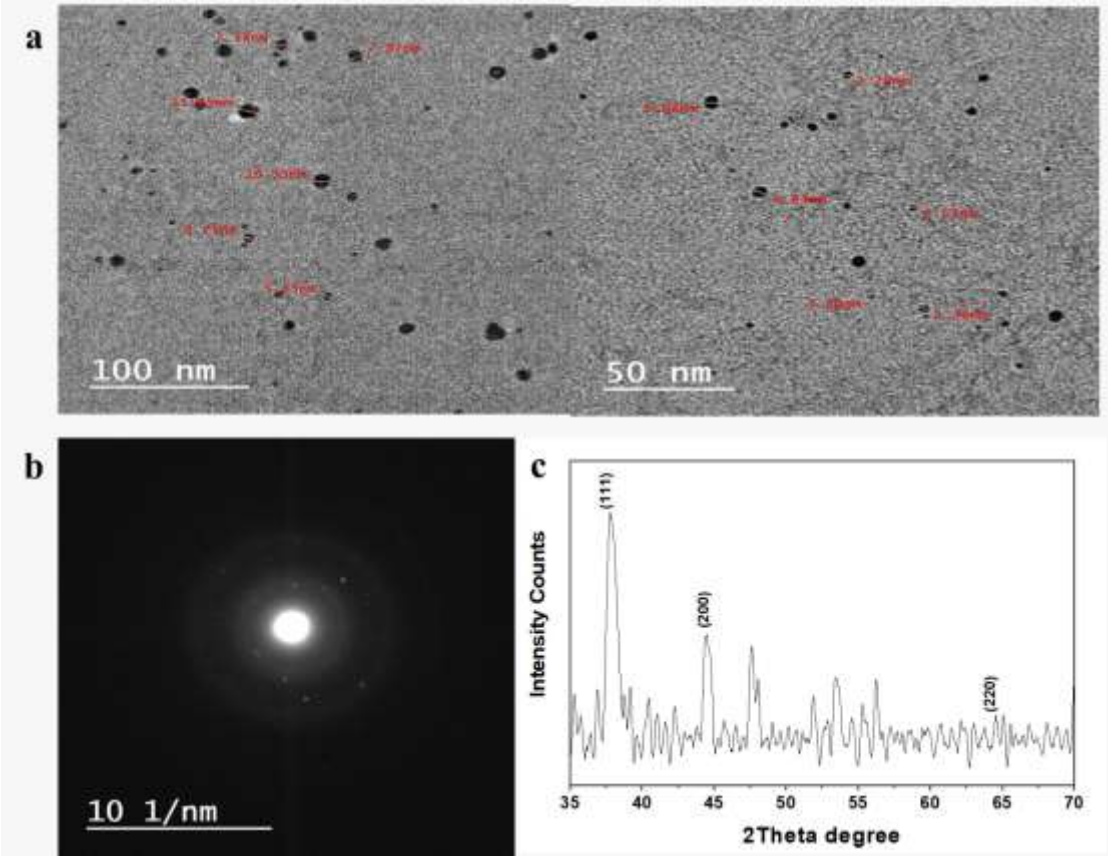

Fig. (3): The morphology and size of the biosynthesized AgNPs by *W. onychis*; **(a)** TEM micrograph, **(b)** TEM Selected area electron diffraction (SAED) image, **(c)** XRD pattern.

Effect of chemical and physical conditions on yeast growth and production of AgNPs

Incubation periods

The samples were collected during various incubation periods. On the first day, the yeast growth accelerated, resulting in an average OD_{600} of 2.1 after 24 hours, compared to an average of 1.84 after 12 hours. In the second and third days, the growth accelerated slowly; in the fourth and fifth days, it stabilized with an average OD_{600}=2.18, and the trend shifted further in the direction of the stationary phase. In the sixth and seventh days, it grew once more, reaching OD_{600}=2.43 in seventh day (**Fig. 4a)** which demonstrates how the yeast growth increase as the incubation period increased.

The yeast cells that were harvested from each incubation period were used to biosynthesize silver nanoparticles, and it was discovered that the amount of AgNPs produced increased as the growth incubation period increased. Seven days is the ideal incubation period for yeast cells to produce a larger quantity of AgNPs **(Fig. 4b, c)**.

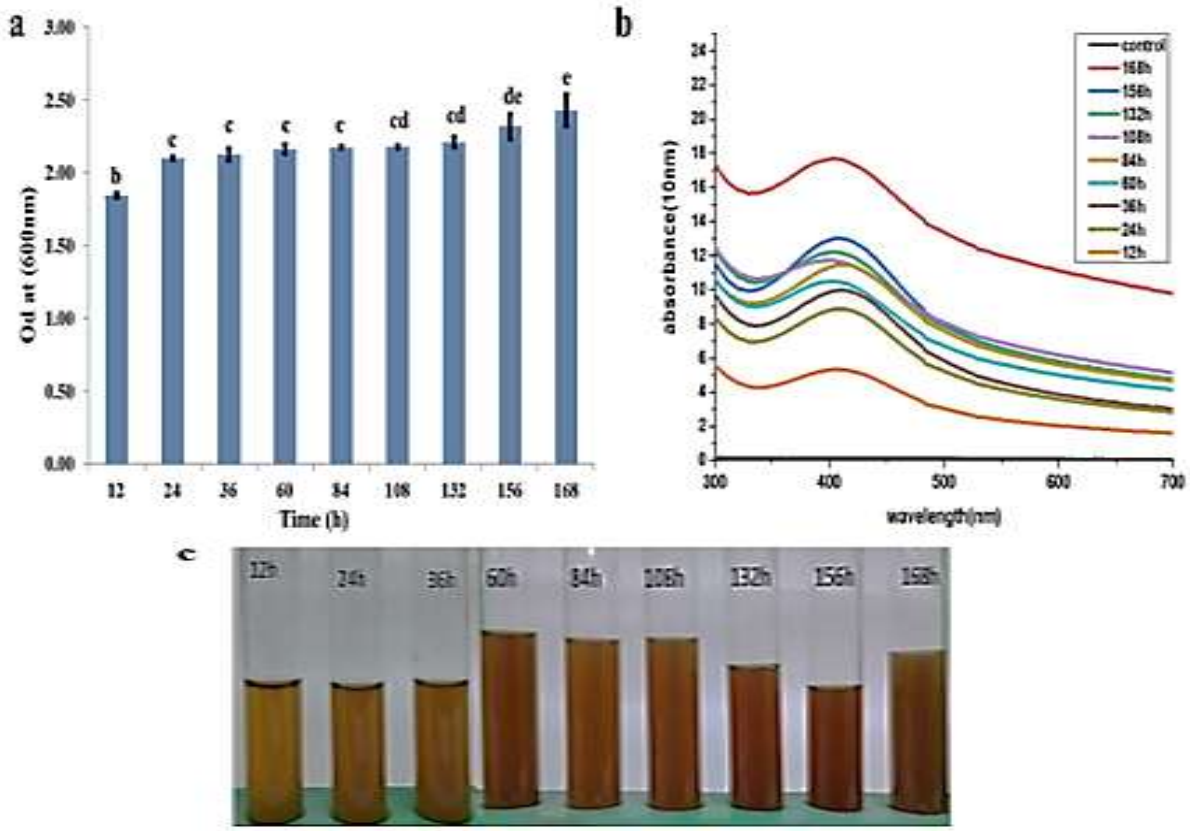

Fig. (4): Effect of incubation period on the yeast growth and silver nanoparticles production by *W. onychis* MSN **(a)** OD of growth cells at 600nm on spectrophotometer after different incubation time, the mean with different letters are significant **($p<0.05$)**; **(b)** UV–vis absorption spectrum for biosynthesized AgNPs using collected yeast cells of samples at different incubation times; and **(c)** Colloidal solution containing silver nanoparticles produced by collected yeast cells from samples of different incubation times.

Growth Media

To investigate each culture medium's impact on the growth of a yeast isolate and AgNPs synthesis, five different types were chosen. It was found that PDB was the ideal medium for producing AgNPs, whereas YPD broth medium was the most suitable culture medium for promoting the yeast's maximal growth **(Fig. 5)**. The highest growth was observed with YPD broth medium (OD600 ≈ 2.6), followed by MYGP broth medium (OD600 ≈ 2.5), and PDB (OD600 ≈ 2.0) **(Fig. 5a)**. Despite the maximum cell growth on YPD media, the collected yeast cells from PDB recorded the highest yield of silver nanoparticles. While the lowest yield of AgNPs were produced by yeast cells from MCD, and none were produced by the cells in DOX media due to the extremely low biomass **(Fig. 5b, c)**.

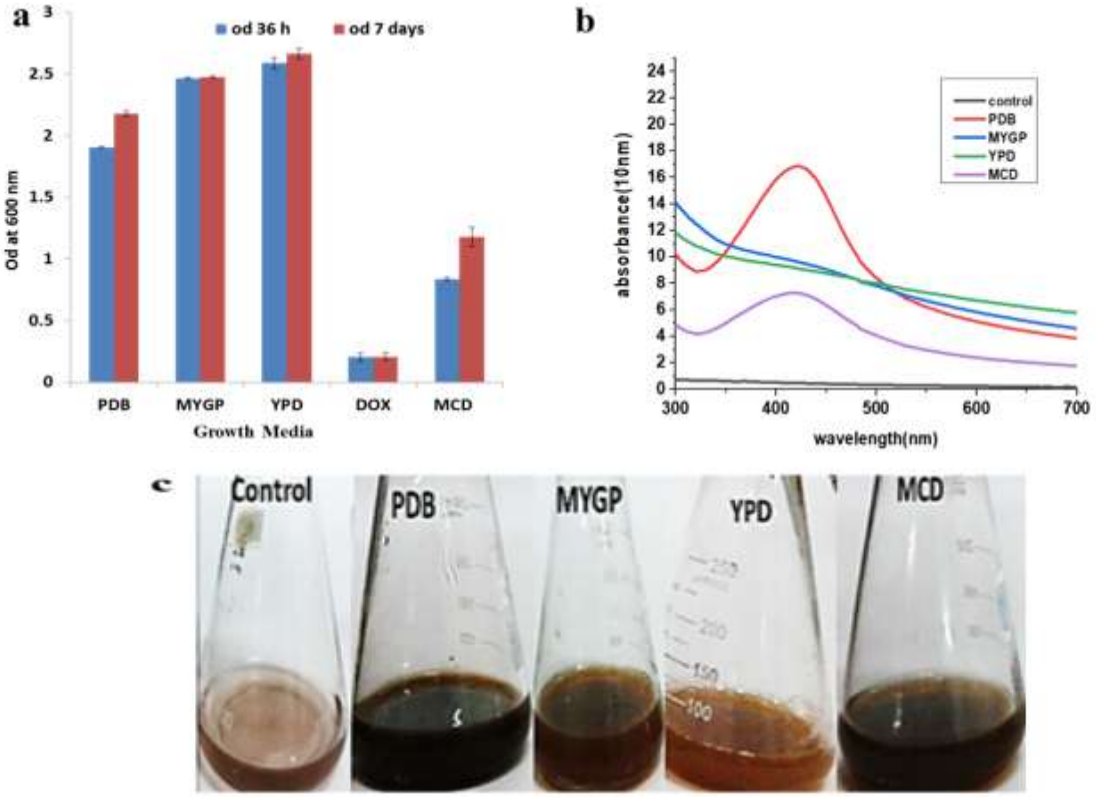

Fig. (5): Effect of different media composition on yeast growth and production of AgNPs by *W. onychis* MSN **(a)** Average OD of growth cells at 600nm on spectrophotometer after 36 h and 7 days; (**b)** UV–vis absorption spectrum for biosynthesized silver nanoparticles by yeast cells after 7 days from growth; and **(c)** Colloidal silver nanoparticle solution was biosynthesized by collected yeast cells from different culture media.

pH Parameter

The quantity of silver nanoparticles produced as well as the growth of yeast cells were evaluated in different the hydrogen ion concentration in the medium. Variation in yeast growth (OD_{600}) was seen at pH values of 4, 5, 6, 7, and 8. After seven days of incubation, the yeast cells' growth reached its peak at pH 8 ($OD_{600} \approx 2.64$), and its lowest point was at pH 4 ($OD_{600} \approx 2.5$) **(Fig. 6a)**. Additionally, the results demonstrated that the yeast cells from the culture media adjusted at initial pH 8 could produce more AgNPs than those that were harvested from the medium of pH 4 (**Fig. 6b)**. This obviously refers to the role of pH in enhancing the synthesis of AgNPs

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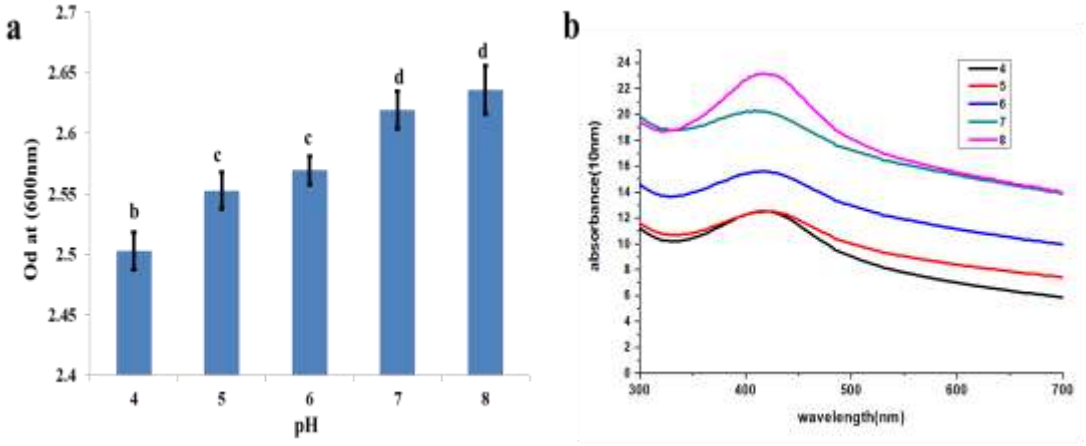

Fig. (6): Effect of different pH degrees on the growth and AgNPs synthesis by *W. onychis* **(a)** OD_{600} yeast growth at different pH after 7days from incubation and **(b)** UV–vis absorption spectrum for biosynthesized AgNPs using separated yeast cells from cultures medium at different pH. Mean with different letters is significant ($P < 0.05$).

Temperature

The effect of temperature on the yeast growth was examined by inoculating the active yeast suspension into PDB media and incubation at 25, 30, and 35°C. The growth of yeast and quantity of AgNPs produced were significantly impacted by the yeast cells' incubation temperature. The growth of the yeast cells was maximum at 30°C (OD_{600} =2.55) and minimum at 25°C (OD_{600} = 1.94) after 7 days of incubation (**Fig. 7a)**. The results also showed that the yeast cells from the treatment of 30°C have produced the highest yield of AgNPs while the lowest yield was observed with the cells of 25°C (**Fig. 7b)**. This obviously could mean that when the incubation temperature raises, the produced biomass cells' reduction potency increases, which in turn increases the formation of AgNPs. Nevertheless, the generation of AgNPs decreased as the incubation temperature rose over 30°C.

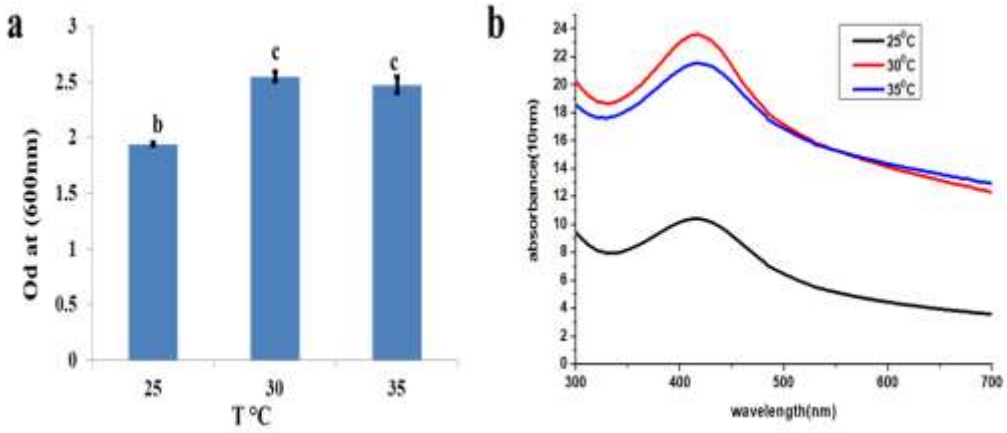

Fig. (7): Effect of different temperature degrees on the growth and AgNPs synthesis by *W. onychis* **(a)** OD_{600} yeast growth at different incubation temperature and **(b)** UV–vis absorption spectrum for biosynthesized AgNPs using separated yeast cells from cultures medium at different incubation temperature. Mean with different letters is significant ($P < 0.05$).

Effect of different concentration of NaCl, KH_2PO_4 and $CaCl_2$ and metal ions ($CoCl_2$, $FeCl_3$ and $MgSO_4$) on the growth and silver nanoparticles production.

The growth of yeast and the quantity of AgNPs produced changed greatly with adding varying concentrations of NaCl, KH_2PO_4, $CaCl_2$, $MgSO_4$, $CoCl_2$, and $FeCl_3$ to the culture media. In case of NaCl, the supplementation of NaCl into the culture media effect negatively on the yeast growth **(Fig. 8a)** as well as AgNPs production **(Fig. 9a)**. On the other hand, 2 PPM of $CaCl_2$, 1 PPM of KH_2PO_4, and 2 PPM of $MgSO_4$ enhance yeast growth compared with the control **(Fig. 8b,c)**. Based on the concentration of $CoCl_2$ and $FeCl_3$, the highest growth of yeast cells was observed at 1 PPM of $CoCl_2$ (OD_{600}=2.39) and (1 PPM and 1.5 PPM) of $FeCl_3$ (OD_{600}=2.36), while the lowest growth was found at 1.5 ppm of $CoCl_2$ and 0.1 PPM of $FeCl_3$ (OD_{600}=2.32) (**Fig. 8d)**. Concerning AgNPs production the metals addition affected significantly on the nanoparticles synthesis (Fig. 9). The highest production rate was observed with 2 PPM of $CaCl_2$ (**Fig. 9b)**, 1 PPM of KH_2PO_4 (**Fig. 9c)**, 2 PPM of $MgSO_4$ (**Fig. 9d)**, 1 PPM of $CoCl_2$ (**Fig. 9e)** and 1.5 PPM of $FeCl_3$ (**Fig. 9f)**.

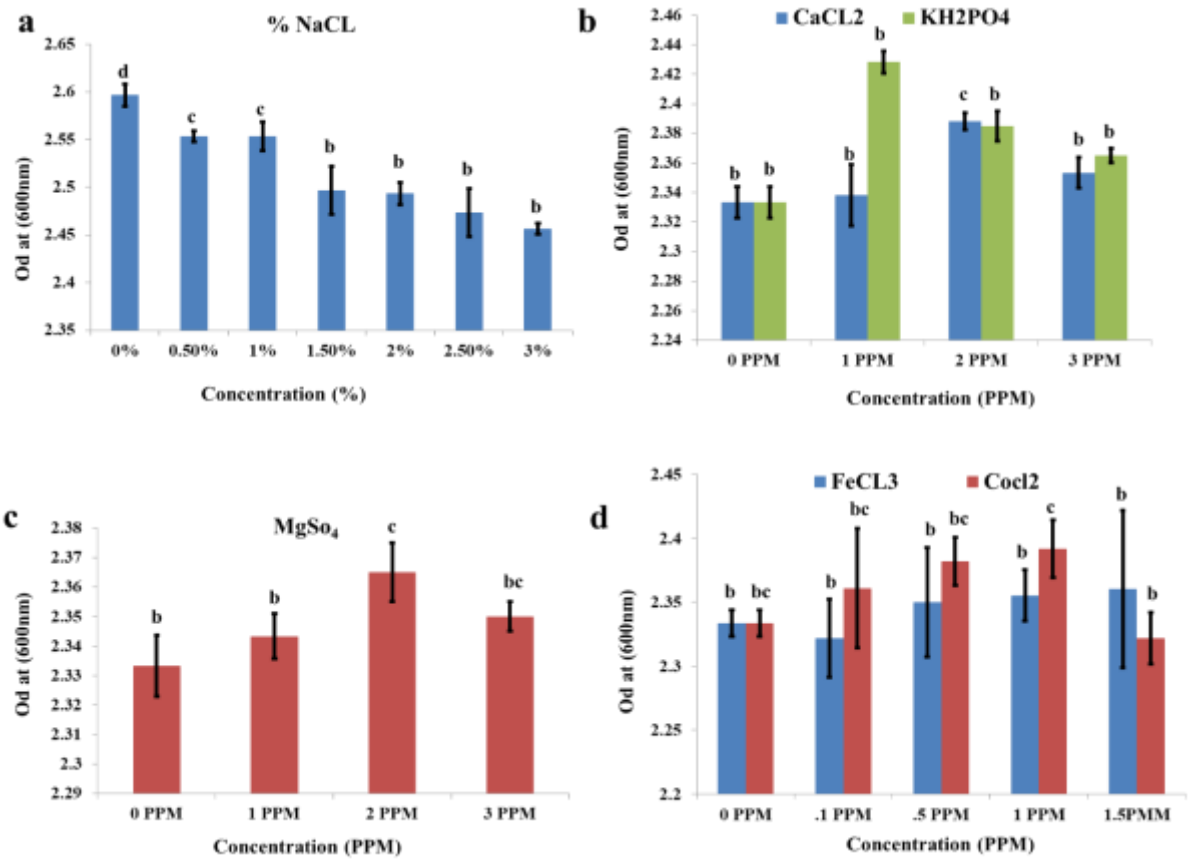

Fig. (8): Effect of different concentrations of different metals ion on the growth of *W. onychis* **(a)** NaCl; **(b)** $CaCl_2$ and KH_2PO_4; **(c)** $MgSo_4$; and **(d)** $FeCl_3$ and $CoCl_2$. The data are the average of three replicates, and the mean with different letters is significant ($P < 0.05$).

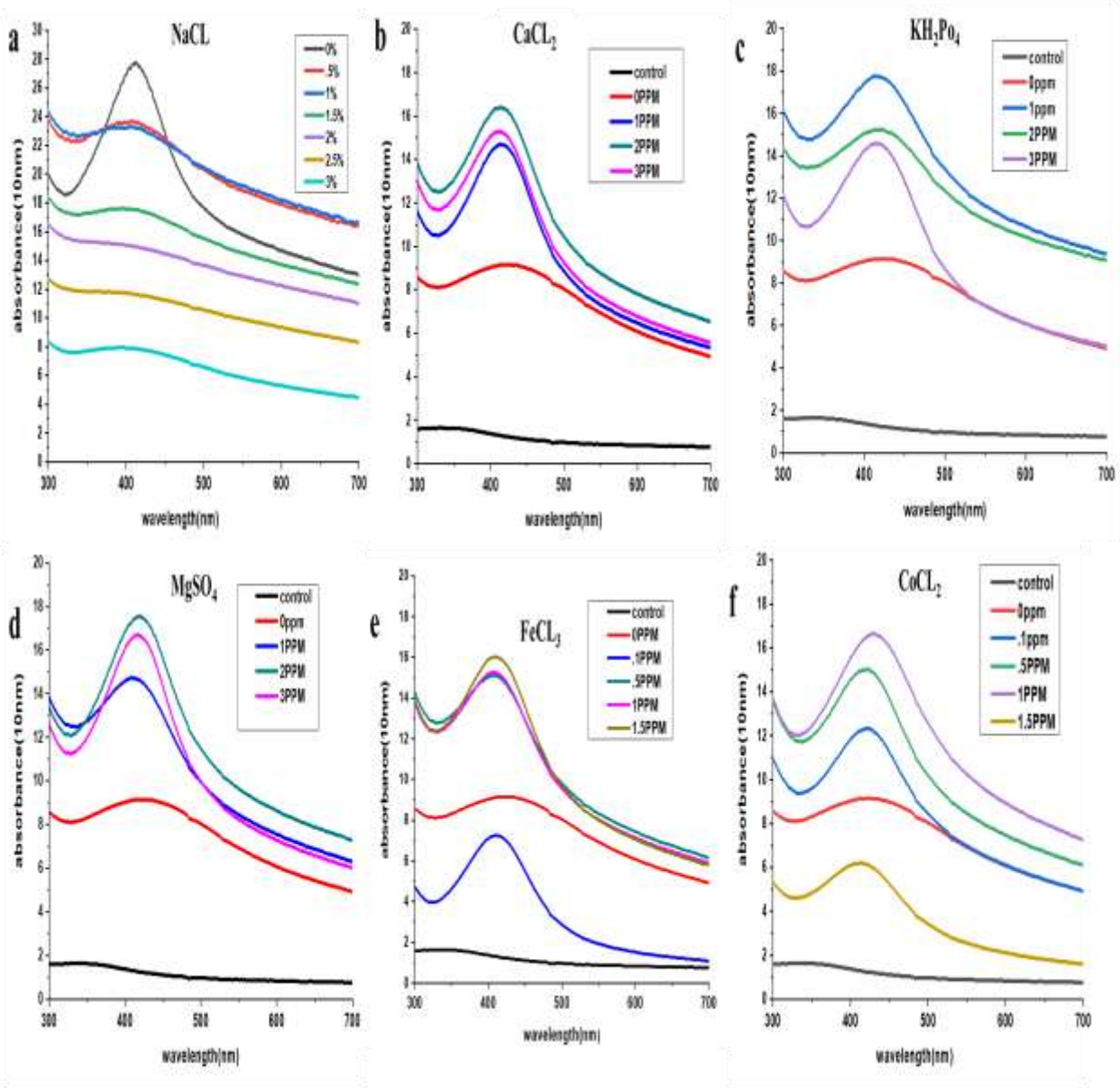

Fig. (9): UV–vis absorption spectrum for biosynthesized AgNPs using collected yeast cells from culture media with different concentrations of metals ions. **(a)** NaCl; **(b)** $CaCl_2$; **(c)** KH_2PO_4; **(d)** $MgSO_4$; **(e)** $FeCl_3$ and **(f)** $CoCl_2$.

Discussion

Microorganisms consider as a golden door for the production of oils, carotenoids **(Elfeky *et al.*, 2019 & 2020)**, antimicrobial compounds **(Zayed *et al.*, 2022; Elfeky *et al.*, 2023**; **Gharieb *et al.*, 2024)**, industrial enzymes **(Elmahmoudy *et al.*, 2021)**, and nanometals **(Khodashenas & Ghorbani, 2016).** The sensitivity and specificity of the nitrate reductase assay (NRA) are significant features, particularly in relation to microbial metabolism and the production of silver nanoparticles (AgNPs) **(Khodashenas & Ghorbani, 2016).**

Understanding these parameters can help assess the reliability of the assay in detecting nitrate reduction, which is a key step in various biochemical processes,

including AgNPs synthesis **(Gupta *et al.*, 2011)**. According to our findings, not every strain that produced silver nanoparticles had a positive nitrate reductase test result. **Zahran *et al.*, (2013)** found that three yeast strains—*Saccharomyces cerevisiae* (Baker's yeast), *Rhodotorula glutinis*, and *Geotrichum candidum*—use yeast secreted proteins and enzymes which could participate in the biosynthesis of AgNPs. **Jalal *et al.*, (2018)** found that protein binds to and stabilizes AgNPs that are biosynthesised by *Candida glabrata*. A link between the concentration of silver nanoparticles and the nitrate reductase enzyme's activity was reported by **Fernández *et al.*, (2016)**. It was found that the *Rhodotorula glutinis* supernatant was less successful than the *Cryptococcus laurentii* supernatant for the synthesis of silver nanoparticles, even though both strains are capable of producing the enzyme. Thus, we may conclude that the nitrate reductase enzyme's synthesis is one of the factors linked to yeasts' ability to manufacture silver nanoparticles; however other factors may also contribute to the generation of silver nanoparticles in greater amounts.

The mechanisms of AgNPs production are widely studied. It has been suggested that the presence of biomolecules such proteins, amino acids, enzymes, vitamins, and polysaccharides in the biomass or supernatant of microbes may cause acting as bioreductants and capping agents **(Gudikandula *et al.*, 2017; Jalal *et al.*, 2018)**. However, the presence of the enzymes nicotinamide adenine dinucleotide (NADH) and NADH-dependent nitrate reductase is primarily responsible for the most widely acknowledged process for the manufacture of AgNPs and other nanoparticles by microorganisms **(Ahmad *et al.*, 2003; Kumar *et al.*, 2007; Kalimuthu *et al.*, 2008)**. The present results would suggest that the enzyme not only interfered with the reaction but other biomolecules such as NADPH are responsible for the AgNPs synthesis**.** The environmental conditions, both physical and chemical, significantly influence the growth of yeast and its metabolic activity **(Rose, 1989)**, thereby impacting its capacity to produce silver nanoparticles. As a result, research was done to discover how different culture conditions affected *W. onychis'* silver nanoparticle biosynthesis. Different culture media, incubation periods, pH values, temperature, different concentration of (NaCl, KH_2PO_4 and $CaCl_2$) and metal ion concentrations ($CoCl_2$, $FeCl_3$, and $MgSO_4$) were studied.

Our findings indicated that the proliferation of yeast cells and the synthesis of AgNPs peaked after 7 days. Our observations align with the augmented proliferation of yeast cells

identified by **Naghavi *et al.*, (2013)**, indicating that as the incubation duration extends, the rate of AgNPs production from yeast biomass consistently intensifies, reaching its zenith after 7 days. This is in opposition to the results of **Khleifat *et al.*, (2022)**, which demonstrated that the filtrate from the biomass of *Rhizopus stolonifera* produce the highest intensity of the AgNPs solution brownish color after 18 hours of growth and the spectrophotometric scan showed that prolonged growth times for *R. stolonifer* MR11 produced biomass with a lower reduction ability. According to other reports, the silver nanoparticles were biosynthesized after 120 hours of biomass growth of *Bipolaris tetramera*, *Aspergillus terreus* HA1N and *Penicillium expansum* HA2N **(Fatima *et al.*, 2015; Ammar & El-Desouky, 2016)**. The kind of microbial strain used affected the size, stability, and dispersion characteristics of the synthesized nanoparticles **(Prakasham *et al.*, 2012)**.

Our finding indicates that the different culture media effect significantly on both yeast growth and AgNPs synthesis. PDB was the optimum media for both variables. Potato extract is an effective source of nitrogen for fungus, yeast, and bacteria, and is abundant in vitamins and minerals which encourage the growth of microorganisms, which may be the cause of the maximum AgNPs production. The nutrient broth was the best medium for the production of silver nanoparticles by *Serratia marcescens* ssp. *sakuensis* **(Nader *et al.*, 2020)**. **Khleifat *et al.* (2022)** found that AgNPs biosynthesis was influenced by the growth media's chemical makeup. Similar results were discovered by **Saxena *et al.*, (2016)**, who observed that the maximum AgNPs synthesis was improved in the fungal biomass of *Sclerotinia sclerotiorum* MTCC 8785 cultivated in PDB. This could be because PDB contains components that promote improved fungal growth and increase the amount of reducing agent necessary for the reduction of silver ions **(Birla *et al.*, 2013)**. Therefore, it is possible to consider that the media composition can affect the internal composition of the cells and their ability to produce silver nanoparticles.

The current study's findings reported that the studied yeast can survive on alkaline media which are consistent with those reported by **(Kurita & Yamazaki, 2002)**. The authors reported that the salt-tolerant yeast *Debaryomyces hansenii* IFO 10939 can thrive at pH 10.0. It was possible for IFO 10939 to keep intracellular pH stable.

Also, the results showed that the growth of the yeast cells was maximum at 30°C and the collected yeast cells from the treatment of 30°C have produced the highest yield of AgNPs. According to earlier research, growing *Aspergillus* sp.

at 32°C leads to the optimal production of silver nanoparticles **(Fouda *et al.*, 2017)**. An important area of research is the impact of different concentrations of NaCL, KH_2PO_4 and $CaCL_2$ and metal ions ($CoCL_2$, $FeCL_3$ and $MgSO_4$) on yeast growth and silver nanoparticle (AgNPs) production. Lower NaCl concentrations (0% to 1%), according to NaCl, typically motivate yeast growth, improving osmotic equilibrium. Higher concentrations (1.5% to 3%), which cause dehydration effects on yeast cells, may cause osmotic stress and lower cell viability and growth rates **(Corte *et al.*, 2006; Shokoohi *et al.*, 2016).** In comparison to previously published results on *Debaryomyces hansenii* (*D. hansenii*), where the highest growth is observed at 0% (w/v) NaCl **(Masoud & Jakobsen, 2005).**

Our results demonstrate that the *Wickerhamomyces onychis* strain exhibit optimal growth and the highest AgNPs production at 0% (w/v) NaCl. On the other hand, the results of **Zhang *et al.*, (2020)** indicate that the majority of *D. hansenii* strains from cheese brine grow best at 4% (w/v) NaCl.

Trace elements supplementation in the medium has a significant impact on the production of extracellular enzymes **(Reddy & Kanwal, 2022)**. For optimum development, metabolism, and high AgNPs production, yeasts need a variety of metals. Metal ions act as co-factors for important enzymes and proteins for yeast growth and high production of AgNPs. Generally speaking, metal ions can affect a number of critical characteristics, such as stress tolerance, enzyme activity, viability, and yeast growth, during the metabolic processes involved in the production of AgNPs **(Chen *et al.*, 2021; Sun *et al.*, 2022).** Therefore, it is necessary to screen vital mineral nutrients for high production of AgNPs. According to **Williams (1970)**, cationic nutrients potassium, magnesium, and calcium are crucial for the structural and functional functions of microorganisms' cells. All living things need iron as a micronutrient because it is involved in biochemical processes like the transportation of oxygen, the storage of molecules, and the catalysis of enzymes, all of which depend on the redox reaction for energy production and metabolism **(Bahafid *et al.*, 2017)**, and the utilization of cobalt ions for the synthesis of vitamin B12 (cyanocobalamine) **(Venkateshwar *et al.*, 2010).**

We do not yet know the exact mechanisms underlying the effects of different concentrations of NaCl, KH_2PO_4, $CaCl_2$, and metal ions ($CoCl_2$, $FeCl_3$, and $MgSO_4$) on *Wickerhamomyces onychis* growth and silver nanoparticle formation from collected biomass.

Conclusion

The relationship between nitrate reductase activity and the synthesis of silver nanoparticles (AgNPs) by yeast isolates, as well as the influence of pH, temperature, and salinity on the growth of *Wickerhamomyces onychis* and the production of AgNPs, are currently the main aim of this study. It was looked into whether or not thirty-one wild yeasts that were isolated from dry foods and vegetables and identified as AgNPs producers could produce the enzyme nitrate reductase. The data show that while all strains are able to biosynthesize AgNPs, only about eight isolates can produce the nitrate reductase enzyme. Thus, the enzyme nitrate reductase is considered as one of the mechanisms that lead yeasts to biosynthesize silver nanoparticles. The process of synthesizing AgNPs is greatly influenced by both chemical and physical conditions. It was discovered that PDB was the optimum medium for producing AgNPs, while YPD was the best medium for growth. The ideal incubation period for the high production of AgNPs and the growth of cells was seven days. The pH of the media was shown to have a significant impact on AgNPs generation, with a value of 8 being ideal. The ideal temperature for AgNPs production and cell development was found to be 30°C. 0% NaCL (control) was the ideal concentration for biomass production and silver nanoparticles biosynthesis. The ideal values were 2 ppm of ($CaCl_2$ and $MgSo_4$), 1 ppm of (KH_2 Po_4), 1,5 ppm of ($FeCL_3$), and 1 ppm of ($CoCL_2$) for both biomass and nanoparticles production.

According to this study, the production of nitrate reductase by yeasts cannot be regarded as the sole factor involved in the synthesis of silver nanoparticles.

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تقييم دور اختزال النترات والتلاعب بالظروف الغذائية في الإنتاج الحيوي لجزيئات الفضة النانوية بواسطة الخمائرالبرية

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يعد الإنتاج الحيوى لجزيئات الفضة النانوية باستخدام خلايا الخميرة مجالًا جذابًا بين الباحثين، لأنه منخفض التكلفة وغير سام وصديق للبيئة. بحثت هذه الدراسة في العلاقة بين إنتاج الجزيئات الفضة النانوية (AgNPs) بواسطة عزلات الخميرة ونشاط إنزيم اختزال النترات. كما قامت بتقييم تأثير الظروف الفيزيائية والكيميائية المختلفة على نمو *Wickerhamomyces onychis* وإنتاج الجزيئات الفضة النانوية. تم تحديد تركيز النتريت لكل عينة. أظهرت النتيجة أنه على الرغم من أن جميع الخمائر قادرة على الانتاج الحيوى ل(AgNPs)، إلا أن ثمانية فقط أنتجت إنزيم اختزال النترات. أظهرت العلاقة بين انتاج الجزيئات الفضة النانوية وإنزيم اختزال النترات ارتباطًا سلبيًا غير ذي دلالة إحصائية. تأثر نمو *W. onychis* و انتاج الجزيئات الفضة النانوية بشكل كبير بالاختلافات في فترة الحضانة ودرجة الحموضة ودرجة الحرارة وتركيز كلوريد الصوديوم وتركيزات المعادن المختلفة. كان الوسط الأكثر فعالية لنمو *W. onychis* هو وسط YPD، بينما كان وسط PDB هو الأمثل لإنتاج الجزيئات الفضة النانوية. كانت الفترة المثلى لإنتاج جزيئات الفضة النانوية ونمو الخميرة سبعة أيام. أثر مستوى الرقم الهيدروجيني للميديا بشكل واضح على درجة حرارة التوليف لتكوين AgNPs ونمو الخلايا كان ٣٠ درجة مئوية. تم تحديد الظروف المثلى لنمو الخميرة وإنتاج الجزيئات النانوية على أنها درجة حموضة ٨، ٣٠ درجة مئوية، وغياب كلوريد الصوديوم، و٢ جزء في المليون من كلوريد الكالسيوم ($CaCl_2$) وكبريتات المغنيسيوم ($MgSO_4$)، و١ جزء في المليون من فوسفات ثنائي هيدروجين البوتاسيوم (KH_2PO_4)، و١.٥ جزء في المليون من كلوريد الحديد ($FeCl_3$)، و١ جزء في المليون من كلوريد الكوبالت ($CoCl_2$). تساهم هذه النتائج في فهم أفضل لإمكانات الإنتاج الحيوي السريع وغير المكلف للجزيئات النانوية المعدنية في المستقبل.