Rhodotorula glutinis: Extracellular Synthesis of Silver Nanoparticals and Lipase Production Using Food Industry Waste, Molasses

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Abstract In the present study, an attempt has been made to evaluate the enzyme activity inhibition of silver nanoparticles originating from yeasts against a major yeast mediated extracellular enzyme, lipase by *Rhodotorula glutinis*. Yeast was cultivated in molasses medium for lipase production. Crude enzyme was incubated with silver nanoparticles, the enzyme activity was determined after the post treatment with different concentration of nanoparticles. Silver nanoparticles were synthesized by *Rhodotorula glutinis.* The different parameters were optimized for the synthesis of AgNPs. The culture was centrifuged at 72,000 × g for supernatant was mixed with 1 mM $AgNO_3$ solution for the synthesis of AgNPs. All the reaction mixtures were incubated at room temperature for under light. The optical characteristics of the synthesized silver nanoparticles were analysed using UV–Vis spectrophotometer, FTIR, SEM, ZS. Crude enzymes were obtained after the respective incubation period by the respective fungal organism. Nanoparticles treatment and the enzyme activity was evaluated by suitable enzyme quantification assays. Finaly we demonstrated the inhibitor or activator effect of the different concentrations of silver nanopartical on the lipase productivity by *Rhodotorula glutinis.* Enzyme activity of all the tested enzymes was not inhibited in all the tested concentration.

Keywords: *Rhodotorula glutinis,* Lipase, Silver nanopartical.

1. Introduction

Nanotechnology is one of the most active research fields in modern material science and technology. Various kind of nano- structured materials such as nanoparticles[S.Gurunathan,K.Kalishwaralal,R.Vaidyanathan,V .Deepak,S.R.K.Pandian,Muniyandi,N.Hariharan,S.H.Eom. 2009], nano-pores [K. Indira, U. Kamachi Mudali, N.Rajendran.2013,K.Indira,S.Ningshen,U.KamachiMudali,N.Raj endran.2011], nanotubes [K. Indira 2015], etc. are available. An array of physical and chemical methods has been used to synthesize NP of particular shape and size for various applications, but they remain expensive. Biosynthesis of AgNPs are becoming popular day by day using microorganisms[Kim, Soo-Hwan Hyeong-Seon Lee, Deok-Seon Ryu, Soo-Jae Choi, Dong-Seok Lee. 2011]. Metallic silver has been subjected to engineered nanotechnology resulting in some extraordinary novel morphologies and characteristics. Silver nanoparticles (AgNPs) are groups of silver atoms ranging in size, in at least one dimension (typically spherical diameter), from 1 to 100 nm. They have been incorporated into medical products, including wound dressings, catheter coatings and bone cement [Moiemen NS, Shale E, Drysdale KJ, Smith G, Wilson YT, Papini R. 2011]. While their small size makes AgNPs so useful in medicine and industries, it may potentially possess a hazard to human health and the environment. The smaller particles, which provide a much larger surface area to mass ratio, are more reactive and toxic than their bulk counterparts [Cha K, Hong HW, Choi YG, Lee MJ, Park JH, Chae HK, Ryu G, Myung H. 2008]. Previous studies have reported that AgNPs are able to interfere with cellular functions, cause toxic effects and, moreover, may interfere with specific biological systems *in vitro* [Foldbjerg R, Dang DA, Autrup H. 2011]. For the last decades, nanotechnology is a skyrocketing multidisciplinary field of research that interweaves physics, chemistry, bionanoscience, and materials science. Among the nanoparticles, silver nanoparticles have several important applications in the field of bio-labeling, sensors, antimicrobial agents and filters. The silver nanoparticles are capable of purifying drinking water, degrading pesticides and killing human pathogenic bacteria [Duncan, T. V. 2011]. Biological materials such as bacteria, fungus, yeasts, plant extracts, actinomycetes, and some biomolecules have been stated as safe to synthesize of MNPs on extracellular and intercellular level [Bankura, K. P., Maity, D., Mollick, M. M. R., Mondal, D., Bhowmick, B., Bain, M. K., *et al*. 2012, Narayanan, K. B., & Sakthivel, N. 2011]. Lipases are acyl hydrolases and water-soluble enzymes that play a key role in fat digestion by cleaving long-chain triglycerides into polar lipids. Because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipase reaction occurs at the interface between the aqueous and the oil phases [P Reis, K Holmberg, H Watzke, ME Leser, R Miller. 2009]. Lipases are present in microorganisms,

plants and animals [Joseph, B., Ramteke, P. W., Thomas, G. 2008]. Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis [Joseph, B., Ramteke, P. W., Thomas, G., 2008]. This hydrolytic reaction is reversible. In the presence of organic solvents, the enzymes are effective catalysts for various inter-esterification and trans-esterification 7 reactions. Furthermore, microbial lipases show regiospecificity and chiral selectivity [Gupta, R., Rathi, P., Gupta, N., Bradoo, S. 2003]. Especially microbial lipases have different enzymological properties and substrate specificities. Many species of bacteria, yeast and molds are found to produce lipases [Liu, Z., Chi, Z., Wang, L., Li, J. 2008].

2. Material & Method

2.1. Microorganisms:

All of the fermentation experiments were conducted using cultures of *Rhodotorula glutinis*, of Hacettepe University Department of Biotechnology Turkey. The microorganisms were cultivated in Sabouraud agar, which is composed of 10 g/L bacteriological peptone, 40 g/L dextrose and 20 g/L bacteriological agar, incubated at 34 ± 1^0C for 48 h an subsequently maintained at 5 ^{o}C in a refrigerator.

2.2. **Synthesis of silver nanoparticles:** Synthesis of silver nanoparticals from *Rhodotorula glutinis* were carried out. The different parameters: pH, Temperature, Incubation Periods, Supernatant Concentrations (ml), Silver Nitrate concentrations (M) were optimized for the synthesis of AgNPs. The culture was centrifuged at 72,000 × g for supernatant was mixed with 1 mM $AgNO_3$ solution for the synthesis of AgNPs. All the reaction mixtures were incubated at room temperature for under light condition. A colour change from pale yellow to brown occurred for yeasts supernatant in the presence of light. The optical characteristics of the synthesized silver nanoparticles were analysed using UV–Vis spectrophotometer, FTIR, ZS, SEM.

2.3. **Production of Lipase in Molasses Medium:** Production and biochemical characterization of lipase by *Rhodotorula glutinis* in a culture supplemented with molasses. This study reveals the utilization of renewable resource for their cost effective production and influence on the process under various conditions. In the production of lipase, culture conditions and media components are investigated as important parameters. After optimizing the incubation perios, effects of molasses concentrations, medium pH and incubation temperature. Olive oil with various concentrations as a different carbon sources were incorporated into the production medium and lipase activity were determined The cell-free supernatant was recovered by centrifugation at10,000 × *g* for 10 min at 4◦C and used to determine extracellu-lar lipase activity. Lipase activity was quantified by free fatty acid titration. Lipolytic activity was calculated following equaiton:

50 x Expended KOH / 30 = U/ml

2.4. Optimization of SNPs and Lipase

*2.4.*1. Effect of pH on lipase activity and SNPs Synthesis:

The optimum pH was determined by incubating the reaction mixture at various pH values ranging from 3 to 9 at 30^0C. The residual enzyme activity and SNPs synthesis at different pH were measured as described above. Assays were carried out in triplicate.

*2.4.*2. Effect of temperature on lipase activity and SNPs Synthesis:

The optimum temperature was determined by incubating the reaction mixture at temperatures in the range of 25-40^0C at an optimized pH value. The thermostability was determined by measuring the residual activity under standard assay conditions following the pre-incubation of the enzyme solution at 30 - 90^{o}C for 30 min. Assays were carried out in triplicate. The temperature of this reaction was optimized by using different temperatures intervals, where the reaction temperatures was monitored from 20-35^0C. The residual enzyme activity and SNPs Synthesis at different temperature were measured as described above. Assays were carried out in triplicate.

***2.4.*3. Effect of Incubation Periods on lipase activity and SNPs Synthesis:**

The optimum Incubation Periods was determined by incubating the reaction mixture at an optimized pH and temperature value between 1-10 day. The residual enzyme activity and SNPs Synthesis were measured as described above. Assays were carried out in triplicate.

2.4.4. **Effect of Substrate on lipase activity and SNPs Synthesis:**

Activity assays was estimated by using various concentrations of substrate from %1 to %5 (olive oil) as a and various concentrations of substrate (silver nitrate) from 1M to 5M was using for determined SNPs synthesis. The residual enzyme activity and SNPs Synthesis were measured as described above. Assays were carried out in triplicate.

2.4.5. **Concentration ratio of yeast supernatant and silver nitrate:**

Similarly, the concentration ratio of yeast supernatant and silver nitrate was optimized with the increasing concentration of yeast supernatant in optimal silver nitrate solution. The absorbance of the resulting solutions was measured spectrophotometrically.

2.4.6. **Stability study of Synthesised SNPs:**

Further, the optimized reaction solution was kept as such in dark at the room temperature and the stability of the synthesized particles was monitored upto 60 days by using UV–vis spectral analysis.

3. Characterization of silver nanoparticles

Synthesized silver nanoparticles were confirmed by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by UV–vis spectra, at the wavelength of 200–700 nm. Further, the reaction mixture was subjected to centrifugation at 75,000 × g for 30 min, resulting pellet was dissolved in deionized water and filtered through Millipore filter (0.45 μm). An aliquot of this filtrate containing silver nanoparticles were used for FTIR and ZS studies. For electron microscopic studies, 25

µl of sample was sputter coated on copper stub and the images of nanoparticles were studied.

2.4. Enzyme Activity Inhibitions by Synthesized SNPs

Effect of synthesized nanoparticles form *Rhodotorula glutinis* on lipase activity from *Rhodotorula glutinis* was studied. The reaction mixture contained 1.0 ml of Olive oil, 0.5 ml $Cacl_2$, 4.5 ml of 50m M acetate buffer (pH 5.6) and 1.0 ml of crude enzyme and various concentration of SNPs. The reaction was carried out at 30^{o}C for 30 min in a shaking water bath, the reaction mixture was then supplemented with 20 ml ethanol. The amount of oleic was determined by titrating the hydrolysis products with 50mM KOH.

4. Result and Discussion

The results of this study indicate that the microorganisms *Rhodotorula glutinis* yeast is potent microorganism for production of lipase in a medium supplemented with molasses at process conditions studied. Besides that, important environmental pollutant molasses; can use as a cheap carbon source for biotechnological applications**.** Enzyme activity inhibition of *Rhodotorula glutinis* mediated silver nanoparticles against microbial extra cellular enzymes has been studied. Synthesis of silver nanoparticles by algal biomass was characterized16 which revealed a strong broad surface plasmon peak located at 418 nm by UV visible spectrophotometry. Moreover a surface plasmon peak remain in the range of 400-440 nm throughout reaction period. That is suggesting the particles are completely dispensed in the aqueous solution. Particle morphology by scanning electron microscopy showed uniform spherical Nanoparticles in the size range of 37-58 nm.The synthesized Nanoparticles were extremely stable for several months after the reaction which indicated by no formation of aggregates and turbidity which rose the high stability of silver nanoparticle in the reaction mixture. Though, inhibitory effect of heavy metals and other chemical inhibitors on enzyme activity of industrial important enzymes produced from various microorganism, nanoparticles mediated enzyme inhibition studies are very few. In this point of view, synthesized silver nanoparticles were evaluated against fungal mediated extra cellular enzymes under laboratory condition.

4.1. Optimization of different parameters on lipase activity and SNPs Synthesis:

Different parameters were optimized for synthesizing silver nanoparticles and lipase including pH, temperature, incubation periods, concentration of Substrate, concentration ratio of leaf extract and silver nitrate, as shown in Fig.1.

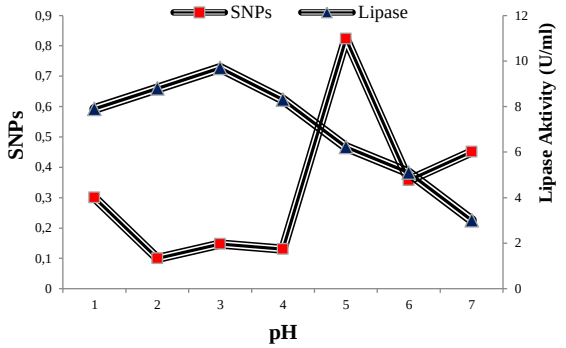

Fig.1. Effect of pH on lipase activity and SNPs Synthesis

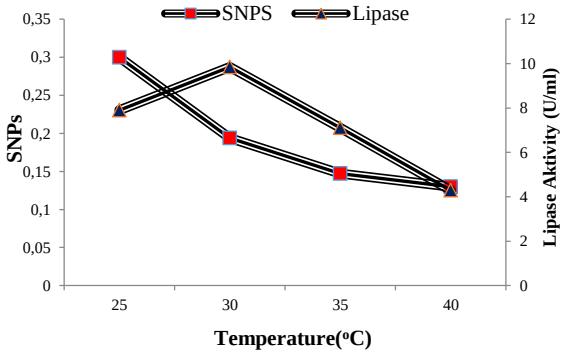

Fig.2. Effect of temperature on lipase activity and SNPs Synthesis

The growth, lipase activity and Synthesized SNPs of the *Rhodotorula glutinis* were determined within the temperature and pH range of 25- 40°C and 3.0 - 9.0, respectively. The highest growth and lipase activity was detected at 30°C and pH 5.0 and of growth medium, respectively . as shown in Fig.2. Enzyme localization was detected as extracellular. Before optimization, *Rhodotorula glutinis* could produce 7.5 U/ml lipase at 30°C and 7.0 pH, after it had produce 9.76 U/ml lipase at 30°C and pH 5. The optimum enzyme production was at 30°C and reduction was observed in enzyme activity above or below 30°C. When the reaction was kept at 60^{0}C for 30 min, the lipase retained almost 9 U/ml activity, whereas only 25% of the maximal activity was lost after incubation at 30-50^{0}C for 30 min. However, the activity was substantially reduced at temperatures higher than 70^{0}C, as shown in Fig.3.The results showed that optimization was succesfuland the optimum pH and maximum enzymatic activity was recorded at pH: 5.0, 9.7 U/ml.

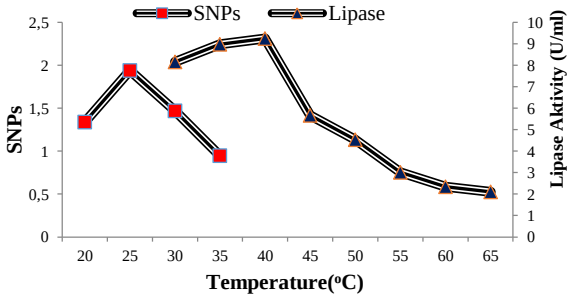

Fig.3. Effect of temperature on lipase stability and SNPs Synthesis (Reaction).

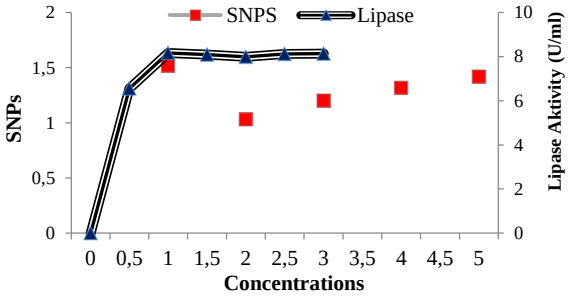

Fig.4. Effect of different concentrations of silver nitrate with yeast supernatant. The reaction was started as soon as the silver nitrate was added into the reaction medium and the formation was observed within 15 min of incubation, The colourless solution was turned to brown colour which indicates the formation of silver nanoparticles Different concentrations of silver nitrate were optimized for the maximum synthesis of silver nanoparticles. Interestingly, 1 mM concentration of silver nitrate supported rapid formation whereas the peak got shifted at 2 mM and 3 mM concentrations, as shown in Fig.4.

These results are in good agreement with the earlier investigations done by Veerasamy *et al.* [Bankura, K. P., Maity, D., Mollick, M. M. R., Mondal, D., Bhowmick, B., Bain, M. K., *et al.* (2012)]. Similarly, different concentrations of yeast supernatant and silver nitrate solution were also optimized for maximum production of silver nanoparticles. Interestingly 25 ml reaction medium containing 2,5 ml of yeast supernatant and 1 mM concentration of silver nitrate solution was turned to brown colour with in 15 min of incubation period, indicating rapid formation of silver nanoparticles, as shown in Fig.5.

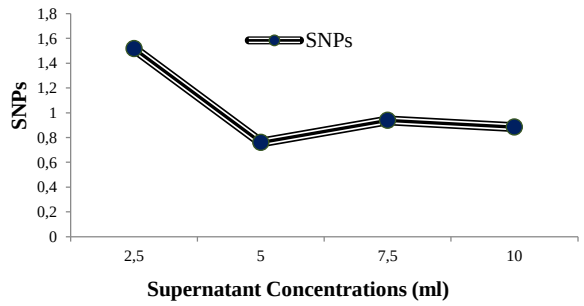

Fig.5. Effect of different concentrations of silver nitrate with yeast supernatant.

Thus the optimized medium supported the maximum formation of silver nanoparticles and the reaction was occurred very rapidly.

Though, inhibitory effect of heavy metals and other chemical inhibitors on enzyme activity of industrial important enzymes produced from various microorganism, nanoparticles mediated enzyme inhibition studies are very few. This work is the first study was conducted to determine the effect of *Rhodotorula glutinis* synthesized nanoparticles on the microbial enzyme production. The results of study showed that, synthesized silver nanoparticles were evaluated against *Rhodotorula glutinis* mediated extra cellular enzymes under laboratory conditions. Enzyme activity of all the tested was not inhibited in all the tested concentration, as shown in Fig.6.

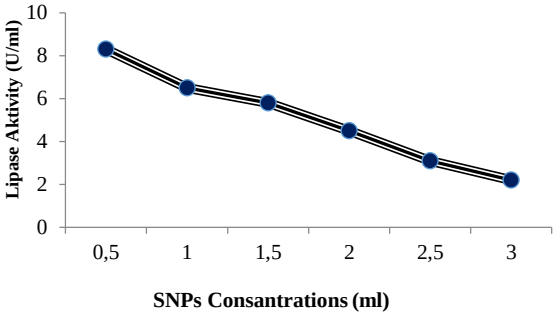

Fig.6. Effect of different concentration of SNPs on lipase activity.

4. Characterization of Silver Nanoparticles

The absorption spectrum of the AgNPs synthesized by *Rhodotorula glutinis* is illustrated as shown in Fig.7. The absorption obtained indicated a strong surface plasmon resonance band maximum at 416 nm, a characteristic peak for silver nanoparticles. The absorption spectrum of silver nanoparticles was observed between 300 nm to 550 nm with peak centered at 410 nm confirmed the formation of silver nanoparticles change in colour to brown was observed due to the excitation of surface plasmon vibrations with silver nanoparticles [V. Gopinath, P. Velusamy, Spectrochim. (2013)].

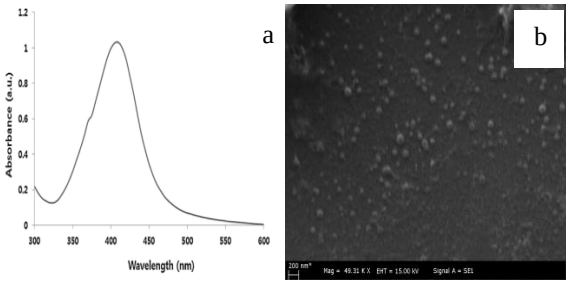

Fig.7.a) UV–Visible spectrum of silver nanoparticles.

b) SEM analysis of biosynthesis nanoparticles with 200 nm scale bar.

The FTIR gives insights about the presence of functional groups in the synthesized silver nanoparticles in order to understand how they transform from simple inorganic silver nitrate to elemental silver due to the effect of different photochemicals that might act in a simultaneous way as reducing, stabilizing and capping agent. The spectrum of FTIR evidently shows the bio fabrication of silver nanoparticles mediated by *Rhodotorula glutinis* at array of absorbance bands from 500 to 4000 cm^{-1}. The FT-IR spectrum analysis for AgNPs represented intense absorption bands at 3271.19, 2921.22, 2846.88, 2573.50, 2161.68, 2021.10, 1974.35, 1634.97, 1568.12, 1516.36, 1453.61, 1386.03, 1034.11and 622.71 cm^{-1} as shown in Fig.8. corresponds to various functional groups.

FT-IR analysis obtained showed the absorption peaks located at 3271.19 cm^{-1} (NH stretch amines), 2921.22-1974.35 cm^{-1} (C-H stretch alkane), 1634.97 cm^{-1} (C=O stretch of amide), 1568.12 1516.36 cm^{-1} (NH bend of amines), 1453.61- 1386.03 cm^{-1} (CH3 bend alkenes), 1034.11 cm^{-1} (CO stretch of carboxylic acid) and 622.71 cm^{-1} (CN stretch of amines). Similar results were reported by [Zarina, A., Nanda, A. (2014), Shahnaz M, Mohd A, Gouri D, Mohammed T, Anima N. (2016)].

The scanning electron microscope analysis was carried out to depict the shape and size of the silver nanoparticles synthesized *Rhodotorula glutinis*.

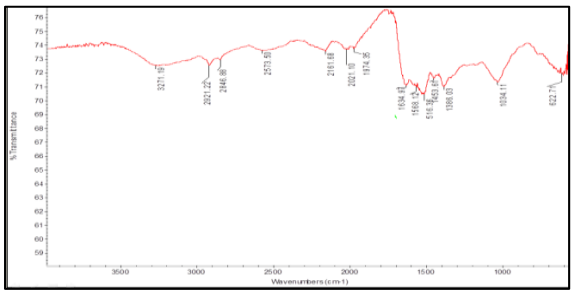

Fig.8. FTIR analysis of silver nanoparticles conferring functional groups.

The scanning electron microscope analysis was carried out to depict the shape and size of the silver nanoparticles synthesized *Rhodotorula glutinis*. The SEM shows that the yeast has tremendous capability to synthesize silver nanoparticles which were well defined separated as much as possible, spherical in shape. The obtained nanoparticles were in the size ranging from 37 to 58 nm as shown in Fig.7.b.

The ZS studies showed the size range of 35 to 78 nm fort he SNPs as shown in Fig.10.

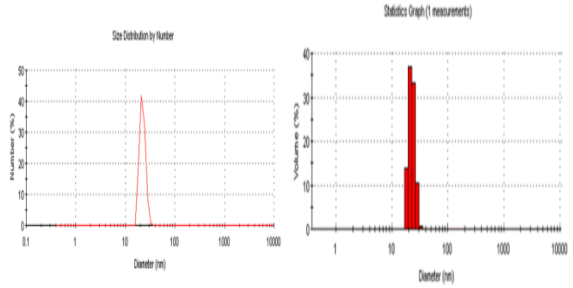

Fig.10. Particles size histogram.

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