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Research Article

MODIFICATION OF *ASPERGILLUS NIGER* ATCC 11414 GROWTH FOR THE ENHANCEMENT OF PROTEASE PRODUCTION BY THE EFFECT OF NATURAL MICROPARTICLES

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Abstract: *Even though fungal proteases are under interest, bulk fungal growth during production decreases the overall mass transfer and consequently the yield. In this study, instead of inorganic microparticles, various organic microparticles were used in the production medium to prevent bulky fungal growth and increase homogeneity. Results showed that all microparticle additions increased the maximum protease activity remarkably and also lowered the required time to reach the highest value. Among organic microparticle addition productions, the highest protease activity was reported as 100,76 U/mL in walnut shells added flasks, which was approximately 1,4 fold higher compared to the highest activity obtained in the control production. It was also reported that microparticle addition increased the homogeneity but also resulted in higher viscosity due to hyphae-type growth. Additionally, evaluation of various storage temperatures showed that produced enzyme lost only its 7% activity at 4°C at the end of 25 days.*

Keywords: *Fungal, protease, microparticle, productivity*

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1. Introduction

Proteases, which break down proteins into their subunits are produced by many organisms from viruses to prokaryotes and eukaryotes. Among them, microorganisms are the main commercial protease producers due to their biochemical diversity and being able to be modified with genetic modifications [1]. Even though *Bacillus* strains dominate the current protease production, interest in fungal proteases, especially in *Aspergillus* strains is increasing due to advantages in terms of high production levels, growth on waste solid materials, low cost, and ease of downstream process step [2,3].

In fungal productions, morphology is one of the most effective factors in yield and productivity. However, the tendency of fungal stains to create bulk growth in the production medium generally ends up with inefficient mass transfer and consequently low product formations. Therefore, preventing bulk fungal growth and the formation of a homogenized production medium is crucial to ensure stable, repeatable, and sustainable productions [4]. Various microparticles such as titanium oxide, aluminum oxide, and talcum were used in fungal productions to prevent bulky growth and aggregation and also to increase homogenization and production yield [5-11]. Even though this technique has many advantages such as low cost, ease of implementation, and not interfering with the fungal metabolism; waste management of these microparticles is a critical environmental problem. Due to the fact that they cannot

be biologically metabolized, disposal of these microparticles creates the necessity for extra costly process steps.

In this study, talcum and various organic microparticles such as walnut shells, pistachio shells, and coffee seeds were evaluated for their ability to enhance protease production with *Aspergillus niger* by controlling fungal growth in shake flask productions.

2. Materials and methods

2.1. Fungal strain and inoculum preparation

A. niger ATCC 11414 was kindly donated by Prof. Dr. Gülşad Uslu Şenel from Firat University, Department of Environmental Engineering, Turkey. The fungal strain was grown on potato dextrose agar (PDA) plates at 25°C for 6 days. The culture was transferred to sterile agar plates biweekly in order to maintain cellular viability. After the incubation, spores were collected by adding 8 mL of sterile 0,1% Tween 80. Suspension, which had approximately 10^8 spores/mL was used to inoculate the production media.

2.2. Shake flask productions

In this study, talcum, walnut shells, pistachio shells, and brewed coffee seeds were used as microparticles. Talcum was used without any pretreatment, whereas walnut shells, pistachio shells, and coffee seeds were first grinded in the coffee grinder and then sieved by using a 750 μm pore size sieve. Thereafter, grinded coffee seeds were brewed with an excessive amount of water to minimize extraction in the protease production medium. After that, all grinded organic microparticles were transferred into 75 mL of double distilled water in 250 mL flasks and autoclaved at 121ºC for 15 minutes to minimize plant-originated extractions. Organic microparticles were then filtered and dried at 55ºC for 8 hours before use. The chemical composition of the protease production medium consisted of 15 g glucose, 3 g meat extract, 2 g KH_2PO_4, 10 g casein, 10 g peptone, and 10 g NaCl per liter of double distilled water. The prepared media was transferred into 250 mL shake flasks with 75 mL working volume and 5% (w/v) microparticles were added to the media. Thereafter, flasks were autoclaved at 121ºC for 15 minutes and after cooling to room temperature, 3% (v/v) inoculum was added into each flask. Flasks were cultured at 25ºC and 180 rpm for 6 days. Samples were collected every 24 hours to measure protease activity.

2.3. Protease activity measurement

Fungal protease activity was measured spectrophotometrically [12] with minor modifications. First, taken samples were centrifuged at 5500 rpm for 10 minutes and then 0.5 mL of supernatant was mixed with 2.5 mL of phosphate buffer (50 mM, pH: 7.0), which contained 0.6% (w/v) casein. After incubation at 25ºC for 20 minutes, the reaction was finalized by the addition of 2.5 mL of 0.44 M trichloroacetic acid. The mixtures were settled for 10 minutes at room temperature and then centrifuged again at 5500 rpm for 10 minutes. From the supernatant, 0.25 mL liquid was first mixed with 1.25 mL of 0.5 M Na_2CO_3 and then 0.25 mL Folin-Ciocalteu phenol. The mixtures were stored at room temperature and dark for 30 minutes. The absorbance was measured at 600 nm using an uninoculated medium as blank. Enzymatic activity value was calculated using a pre-calculated tyrosine standard. One unit of enzyme unit (U) was defined as the amount of enzyme, which is required to release 1 μmol of tyrosine at 25ºC per minute.

2.4. Stability of protease activity under various storage temperatures

Fungal protease, which was produced in walnut shell added flasks at the maximum level, was centrifuged at 5500 rpm for 10 minutes and then supernatants were transferred into the sterile microcentrifuge tubes. Thereafter, tubes were stored at -20, 4, and 25°C conditions, and samples were taken periodically to calculate the stability of activity.

3. Results and discussion

In this study, the effect of talcum and various organic microparticles on protease production by *A. niger* was evaluated. Fungal growth morphology, protease production yield, productivity, also enzyme stability under different temperature storage conditions were evaluated.

Viscosity is a key parameter to be considered in bioprocessing systems, which directly affects mass transfer, aeration and agitation efficiency, and power input [13]. Addition to production medium composition, fungal growth structure is also highly effective on medium rheology and viscosity. At 72 hours of the shake flask production, images were taken from the bottom of the flasks to make an evaluation of fungal growth morphologies (Figure 1). As it can be seen in Figure 1a, a heterogeneous fungal formation occurred in the flask, in which there was not any microparticle added. Lots of small sizes and also big bulk fungal growth structures were observed, which decreases the reliability of stability and repeatability of the productions. On the other hand, the production broth showed clear and not viscous characteristics in the control flask, which provides advantages during separation and purification steps. In all microparticle-added flasks, fungus performed only small or hyphae growth structures. Whereas talcum-added production flasks resulted in a very high viscous broth (Figure 1b), walnut shells added flask (Figure 1c) showed lower resistance to flow. Also, pistachio shells (Figure 1d) and coffee seed shells (Figure 1e) added flasks resulted in mild-level viscous broths.

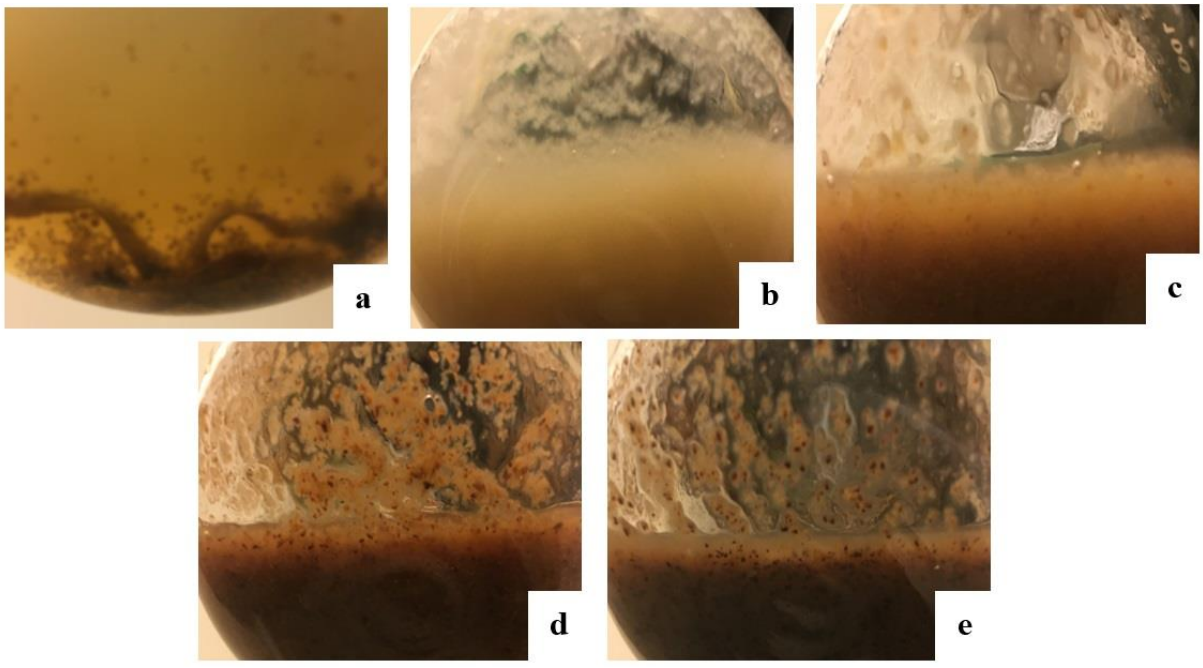

Figure 1. Fungal growth morphologies of *A. niger* with the effect of microparticles (a: Control, b: Talcum addition, c: Walnut shell addition, d: Pistachio shell addition, e: Coffee seed addition)

Fungal protease activities during the production were measured and graphed in Figure 2. It can be clearly seen from Figure 2 that, all microparticle-added flasks resulted in higher maximum protease activities compared to the control. The highest protease activity was calculated as 127,16 U/mL in talcum-added production and the lowest was in coffee seed-added production as 83,16 U/mL. Among organic microparticle-added productions, the maximum protease activity was calculated as 100,76 U/mL in walnut shells added flasks. However, the highest enzyme activity was computed as 73,26 U/mL in the control flask. It was also reported that the highest protease activities were obtained at 120 hours of production in all microparticle-added flasks. Nevertheless, the maximum protease activity was observed at 144 hours of production in the control. These results clearly state that microparticle addition both increased the maximum enzyme activity and also lowered the time to reach the top point, which resulted in higher productivity. Productivity values were calculated as 0,7, 1,74, 0,84, 0,9, and 0,97 U/mL/h for control, talcum added, walnut shell added, pistachio shell added, and coffee seed added productions, respectively. It was also noted that protease activities followed almost the same pattern till 24 h of the production, however lower enzyme activity was measured in coffee seed-added flasks compared to control at 48 h of the fermentation and then activity increased in the further sampling times. This can be explained by the potential negative effect of further released agrochemical byproducts such as phenolics and furans on microbial metabolism [14].

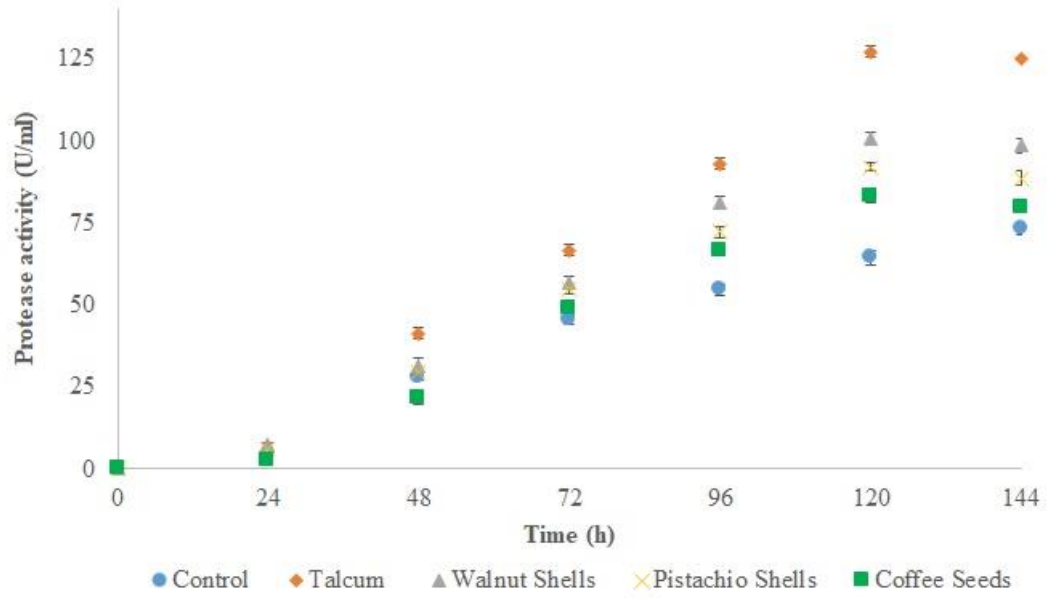

Figure 2. *A. niger* protease activity trends during the production under the effect of various microparticles.

The effect of microparticles on the enhancement of fungal protease production is further discussed and related to various physiochemical properties of the microparticles as shown in Table 1. A comparison of the densities of the microparticles showed that talcum is approximately 5 times heavier than other organic microparticles. Since all microparticles were added to the flasks based on weight per volume, small number of talcum microparticles were in the flask compared to others due to the higher density of talcum. Nevertheless, in comparison based on the particle size, talcum particles were 30 to 75 times smaller than organic microparticles, which potentially resulted in a higher number of talcum particles in the flasks compared to organic microparticles added flasks. Additionally, evaluation of the hardness of microparticles showed that talcum is the softest and coffee seed is the hardest microparticle used in this study. Hardness is an effective factor in fungal growth during especially a long period of

cultivation (144 h) in terms of fragility and preservation of the integrity of the particles. At least but not the less, talcum is not biologically available for fungi, and disposal of it causes serious environmental problems. However, organic microparticles can be disposed of environment without any side effects, and additionally, various nutrients such as sugars, which are released from plant-based wastes can be partially metabolized by the fungi during production [14].

Table 1. Various physicochemical properties of microparticles.

Property	Talcum	Walnut Shells	Pistachio Shells	Coffee Seeds
Density (g/cm^3)	2.58-2.83	0.48-0.52	0.45-0.50	0.56-0.75
Size (µm)	~10	~300-750	~300-750	~300-750
Hardness (Moh)	~1.0	~3.5	~3.5	~5.0
Biological degradation	No	Yes	Yes	Yes

After all, in order to make a more efficient comparison of the effect of organic and inorganic microparticles on fungal productions, optimization and standardization of mechanical, physical, and chemical properties of the particles are required.

Walnut shells added production broth was centrifuged and cell-free supernatants were transferred into the tubes and stored at -20, 4, and 25ºC conditions. As seen in Figure 3, samples, which were stored at 4 and 25ºC temperatures remained their activities at the end of the first day. However, freezing samples caused a sharp decrease (higher than 50%) in the protease activity even on the first day. This can be explained by the adverse effect of ice crystal occurrence on the protein structure of the enzyme [15]. Therefore, cryoprotectant addition to samples before freezing is highly recommended. After the first day, frozen samples almost kept their activity values the same till the end of 25 days. Activity differences between samples, which were kept at 4 and 25ºC started to increase by the third day of storage. With the advantage of minimizing water activity, 4ºC storage conditions resulted in better activity protection compared to 25ºC. By the end of 25 days, protease activity losses were calculated as 7% and 25% for 4 and 25ºC storage conditions, respectively.

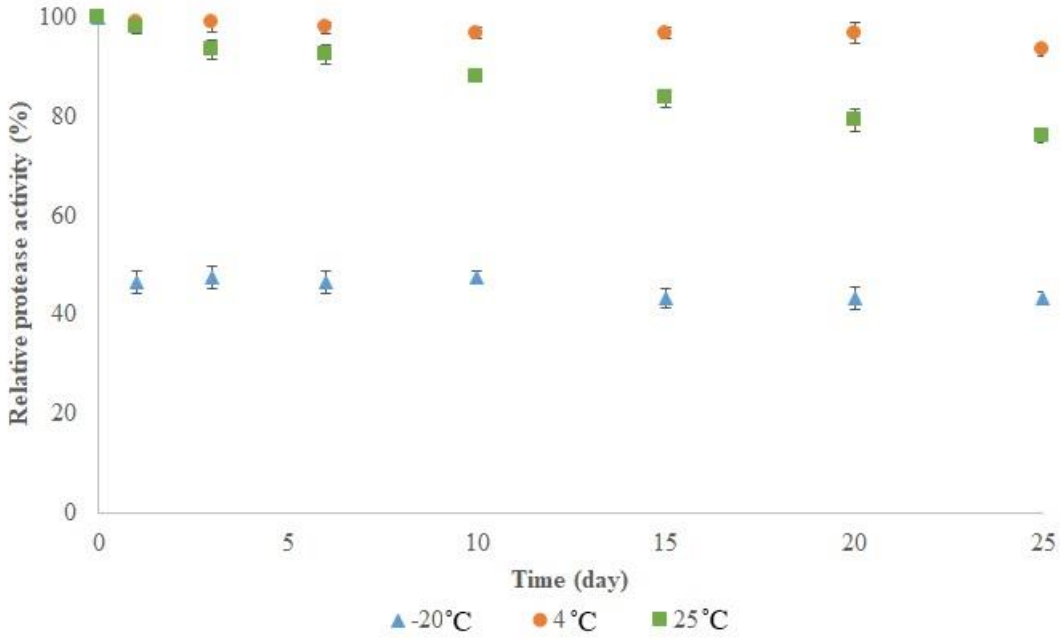

Figure 3. Effect of storage temperature on fungal protease activity

4. Conclusion

In this study, it was clearly shown that organic microparticles are strong substitute candidates for inorganic particles, which can be used for the enhancement of fungal production. The addition of organic microparticles can both increase the highest protease activity and also decrease the time, which is required to reach the maximum activity value. Additionally, it was shown that the mechanical and physicochemical properties of the microparticles should be further discussed and studied in order to provide a better understanding of the overall process. Furthermore, it was reported that produced enzyme can maintain its activity by 93% at the end of 25 days at 4ºC storage conditions.

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I thank Prof. Dr. Gülşad Uslu Şenel from Firat University, Department of Environmental Engineering for providing the fungal strain, which was used in this study, and also Ass. Prof. Dr. Hatice Güneş Özhan, who kindly shared her laboratory with me for my studies at Izmir Biomedicine and Genome Center.

Ethical statement
No ethical statement is needed for this study.

Conflict of interests
As the author, I declare no conflict of interest.

Author contributions
As the single author in this study, I fully contributed to the study conception and design (%100). The author read and approved the final manuscript.

References

[1] Purushothaman, K., Bhat, S.K., Singh, S.A., Marathe, G.K., Rao, A.R.G.A., "Aspartic protease from *Aspergillus niger*: Molecular characterization and interaction with pepstatin A", *International Journal of Biological Macromolecules*, 139, 199-212, 2019. Doi: 10.1016/j.ijbiomac.2019.07.133

[2] Siala, R., Frikha, F., Mhamdi, S., Nasri, M., Kamoun, A.S., "Optimization of acid protease production by *Aspergillus niger* I1 on shrimp peptone using statistical experimental design", *The Scientific World Journal*, 2012. Doi: 10.1100/2012/564932

[3] Yin, L.J., Hsu, T.H., Jiang, S.T., "Characterization of acidic protease from *Aspergillus niger* BCRC 32720", *Journal of Agricultural and Food Chemistry*, 61(3), 662-666, 2013. Doi: 10.1021/jf3041726

[4] Karahalil, E., Coban, H.B., Turhan, I. "A current approach to the control of filamentous fungal growth in media: microparticle enhanced cultivation technique", *Critical Reviews in Biotechnology*, 39(2), 192-201, 2019. Doi: 10.1080/07388551.2018.1531821

[5] Coban, H.B, Demirci, A., "Enhancement and modeling of microparticle-added *Rhizopus oryzae* lactic acid production", *Bioprocess and Biosystems Engineering*, 39(2), 323-330, 2016. Doi: 10.1007/s00449-015-1518-0

[6] Coban, H.B., Demirci, A., Turhan, I., "Microparticle-enhanced *Aspergillus ficuum* phytase production and evaluation of fungal morphology in submerged fermentation", *Bioprocess and Biosystems Engineering*, 38(6):1075-1080, 2015. Doi: 10.1007/s00449-014-1349-4

[7] Coban, H.B., Demirci, A., Turhan, I., "Enhanced *Aspergillus ficuum* phytase production in fed-batch and continuous fermentations in the presence of talcum microparticles", *Bioprocess and Biosystems Engineering*, 38(8), 1431-1436, 2015. Doi: 10.1007/s00449-015-1384-9

[8] Kaup, J.A., Ehrich, K., Pescheck, M., Schrader, J., "Microparticle-enhanced cultivation of filamentous microorganisms: Increased chloroperoxidase formation by *Caldariomyces fumago* as an example", *Biotechnology and Bioengineering*, 99(3):491-498, 2008. Doi: 10.1002/bit.21713

[9] Driouch, H., Hansch, R., Wucherpfennig, T., Krull, R., Wittmann, C., "Improved enzyme production by bio-pellets of *Aspergillus niger*: Targeted morphology engineering using titanate microparticles", *Biotechnology and Bioengineering*, 109(2), 462-471, 2012. Doi: 10.1002/bit.23313

[10] Walisko, R., Krull, R., Schrader, J., Wittmann, C., "Microparticle based morphology engineering of filamentous microorganisms for industrial bio-production", *Biotechnology Letters*, 34(11), 1975-1982, 2012. Doi: 10.1007/s10529-012-0997-1

[11] Driouch, H., Roth, A., Dersch, P., Wittmann, C., "Filamentous fungi in good shape: Microparticles for tailor-made fungal morphology and enhanced enzyme production", *Bioengineered Bugs*, 2(2), 100-104, 2011. Doi:10.4161/bbug.2.2.13757

[12] Vaithanomsat, P., Malapant, T., Apiwattanapiwat, W., "Silk degumming solution as substrate for microbial protease production", *Kasetsart Journal - Natural Science*, 42, 543-551, 2008, Doi:10.1016/j.jbiotec.2008.07.1967

[13] de Jesus, S.S., Neto, J.M., Maciel, R., "Hydrodynamics and mass transfer in bubble column, conventional airlift, stirred airlift and stirred tank bioreactors, using viscous fluid: A comparative study", *Biochemical Engineering Journal*, 118, 70-81, 2017. Doi: 10.1016/j.bej.2016.11.019

[14] Demirel, F., Germec, M., Coban, H.B., Turhan, I., "Optimization of dilute acid pretreatment of barley husk and oat husk and determination of their chemical composition", *Cellulose*, 25(11), 6377-6393, 2018. Doi:10.1007/s10570-018-2022-x

[15] Cao, E.H., Chen, Y.H., Cui, Z.F., Foster, P.R., "Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions", *Biotechnology and Bioengineering*, 82(6), 684-690, 2003. Doi:10.1002/bit.10612