



Short Communication

Vermiculite as a new carrier for extracellular protease production by *Aspergillus* spp. under solid-state fermentation



Alexander A. Osmolovskiy^{a,*}, Elizaveta A. Popova^a, Valeriana G. Kreyer^a,
Nina A. Baranova^a, Nikolay S. Egorov^b

^aDepartment of Microbiology, Faculty of Biology, Lomonosov Moscow State University, Moscow, Russian Federation

^bInternational Biotechnological Center, Lomonosov Moscow State University, Moscow, Russian Federation

ARTICLE INFO

Article history:

Received 7 June 2020

Received in revised form 22 November 2020

Accepted 28 November 2020

ABSTRACT

A new method has been developed to increase the productivity of aspergilli - producers of extracellular proteinases based on their cultivation on vermiculite under solid-state fermentation conditions. The productivity of the mycelium *Aspergillus ochraceus* L-1 and *Aspergillus ustus* 1 was 3–18 times higher not only in comparison with submerged cultivation, but also in comparison with growth on other carriers studied under solid-state fermentation conditions. Vermiculite can be considered as a new promising carrier for solid-state fermentation of micromycetes.

© 2020 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Solid-state fermentation (SSF) is a widespread technique for extracellular enzyme production by filamentous fungi based on their cultivation on moistened particles to which any liquid of the system (water or nutrient medium) is completely bound [1,2,17]. SSF is most favorable for filamentous fungi and has considerable advantages over traditional submerged fermentation (SmF) for extracellular enzyme production. In particular, filamentous fungi under SSF conditions yield 2- to 10-fold more hydrolases as compared with submerged fermentation. Such overproduction is determined by the fact that micromycetes develop under conditions that are closer to their natural growth conditions [3–6]. In SSF a wider range of micromycete genes are expressed, including genes encoding enzymes. Certain enzymes are produced only under these conditions and obtaining them by SmF is not possible. The differences in physicochemical properties of the enzymes synthesized by micromycetes in SSF from those produced in SmF are of considerable scientific interest. It was shown that the best carriers for SSF are inert carriers, such as polyurethane foam, perlite, amberlite, or silica gel, which show biological and chemical stability and a high water capacity [6,7,17]. However, to obtain specific enzymes in the cultivation of specific producers, it is necessary to work on the selection of a suitable carrier. The carrier for SSF should provide good anchoring of fungal hyphae, a large surface area, and the secretion of a significant amount of

extracellular enzymes. In view of this, studies are emerging presenting different substrates and carriers for SSF [8–10].

One of the most suitable carriers for SSF may be vermiculite. It is a hydrated magnesium aluminum silicate micaceous mineral that is capable of exfoliation and resembles mica in appearance. Vermiculite mines are found in various parts of the world including Australia, Brazil, Bulgaria, Kenya, Russia, South Africa, Uganda, USA and Zimbabwe. Vermiculite, when subjected to heat, exfoliates to form elongated concertina-like particles which are lightweight, incombustible, compressible, highly adsorbent, and non-reactive [11]. Exfoliated vermiculite is used in a wide number of markets including the construction industry (concrete as aggregate, in plaster and premix, and in blocks), in insulation (loose fill, acoustical tiles), in horticulture and agriculture (aggregate, seed germination, soil amendment, fertilizer carrier). It has a high moisture content and porosity, can hold 2–5 times its weight in water [12] Also vermiculite particles are of suitable size for their usage as a carrier in solid-state fermentation.

Proteases of microbial origin prevail on the market and are widely used in biotechnology, as they have several advantages: high activity, ease of purification, thermal stability, possibility of genetic manipulation, and economic profitability. They are successfully used in the laundry industry (for example, alkalase from *Bacillus licheniformis*), as well as in the chemical industry (*Aspergillus*, *Bacillus*, *Pseudomonas*) and medicine. Compared to bacteria, mycelial fungi (micromycetes) are able to secrete a larger spectrum of extracellular proteases, which play an important role in commerce. Proteases of fungal origin are able to function in a wider pH range (pH 4–11) and have a broader substrate specificity, which is why they are used in medical practice as therapeutic

* Corresponding author.

E-mail address: aosmol@mail.ru (A.A. Osmolovskiy).

agents for the treatment of blood coagulation pathologies, as part of cosmetics, as well as in the food industry for clarifying various beverages (beer, fruit juices, wine), for improving dough consistency or curdling of milk [13]. An important characteristic of a protease producer is the presence of a target type of activity: for collagenases this is collagenolytic activity, for proteinases acting on proteins of the hemostasis system these are fibrinolytic and specific amidolytic activities.

Many proteases are produced by filamentous fungi especially under SSF conditions. Filamentous fungi from genus *Aspergillus* are the most favorable producers of proteases with different properties and specificity.

The aim of this work was to develop a new SSF system for proteases production by *Aspergillus* with the usage of vermiculite.

2. Materials and methods

2.1. Study object

The study was performed with microscopic filamentous fungi *Aspergillus ochraceus* L-1 and *Aspergillus ustus* 1, selected in earlier studies as the most active producers of extracellular proteinases [14]. To obtain the inoculation material, micromycetes were grown in test tubes on wort agar slants at 25 °C for 7 days.

2.2. Solid-state fermentation of micromycetes

Sterilized polyurethane foam (in the form of cubes of 5.0 × 5.0 × 5.0 mm, porosity 16 %, moisture content by volume 12 %), perlite (particle size up to 2 mm, porosity 26 %, moisture content by volume 40 %), silica gel (particle size up to 1.5 mm, porosity 30 %, moisture content by volume 60 %) and vermiculite (particle size up to 3.5 mm, porosity 42 %, moisture content by volume 53 %) were added into Eppendorf® Cell Culture Flasks T-75 with a filter cap. To prevent overheating of systems, in the case of polyurethane foam, the height of the carrier layer did not exceed 0.5 cm, in the cases of other carriers - 0.35 cm. Flasks were poured with a sterile nutrient medium with the same composition as we used in previous studies [7,14]: 3.5 % glucose, 0.1 % starch, 0.5 % fish flour hydrolysate, 0.5 % peptone, 0.2 % NaCl, 0.05 % KH₂PO₄, 0.05 % MgSO₄, pH 5.5. The optimum ratio between the amount of carrier, the nutrient medium and the duration of cultivation were determined in

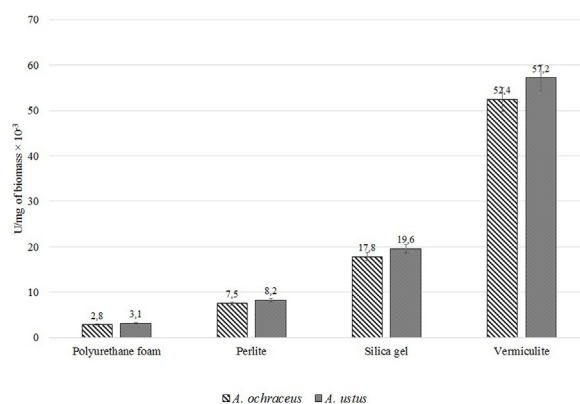


Fig. 2. Productivity of mycelium *A. ochraceus* L-1 and *A. ustus* 1 under SSF conditions (growth on different inert carries).

preliminary experiments, depending on the moisture capacity of each carrier. An amount of growth medium was used so that all of the added liquid was bound by the carriers.

In the experiment with polyurethane foam the amount of the substrate and the volume of the added nutrient medium were 2.5 g and 20 mL respectively; perlite, 5 g and 15 mL; silica gel, 10 g and 15 mL; and vermiculite, 5 g and 25 mL. Inoculation was performed with 1 mL of a spore suspension obtained by washing the spores from the wort agar culture (2.1×10^6 spores/mL). Cultivation of aspergilli was performed under stationary conditions at 28 °C. Proteolytic enzymes were eluted with 0.05 M Tris–HCl buffer, pH 8.2 (suitable buffer for selected micromycetal proteases, [14]). The flasks with polyurethane were supplemented with 50 mL of the buffer; perlite and silica gel, 20 mL; and vermiculite, 35 mL. The amount of added buffer was calculated so that it would completely cover the support layer and the growth surface of the micromycete. After the addition of the buffer, the flasks were incubated on an orbital shaker (200 rpm) for 60 min.

2.3. Biomass determination

The biomass was determined gravimetrically in glass Petri dishes by drying it at 86 °C to a constant weight (Wb) and then calculated as follow: $Wb = W1 - W2 - W3$, where W1 is the weight of

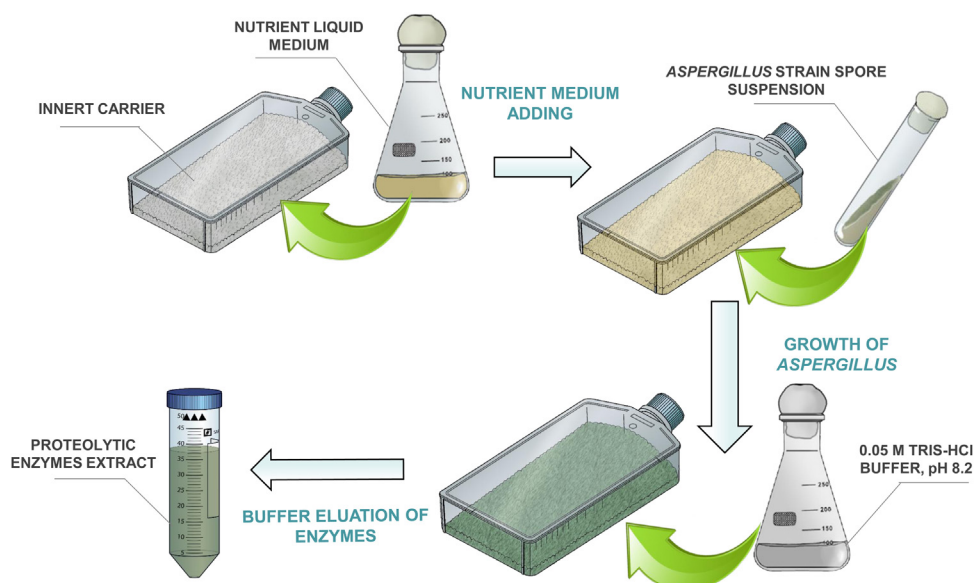


Fig. 1. Laboratory scheme for SSF of aspergilli.

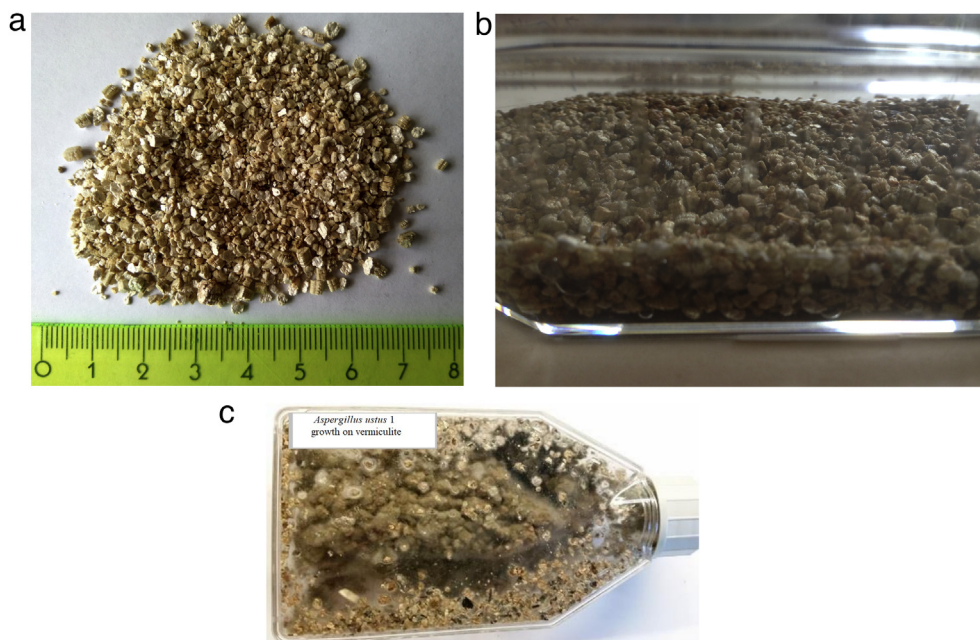


Fig. 3. Uninoculated vermiculite (a), vermiculite moistened with nutrient medium (b) and growth of *Aspergillus ustus 1* under SSF on vermiculite (c).

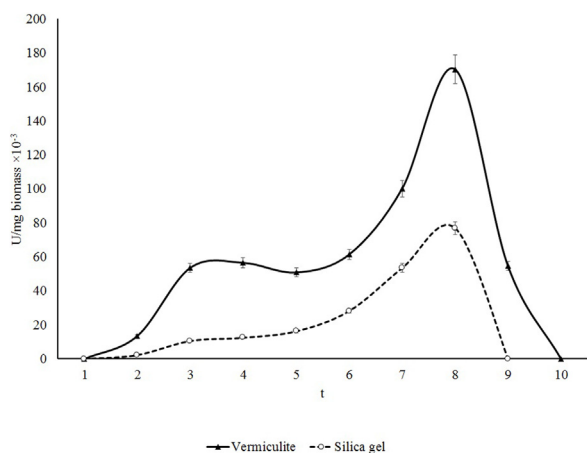


Fig. 4. Dynamics of proteolytic activity of micromycete *A. ochraceus* L-1.

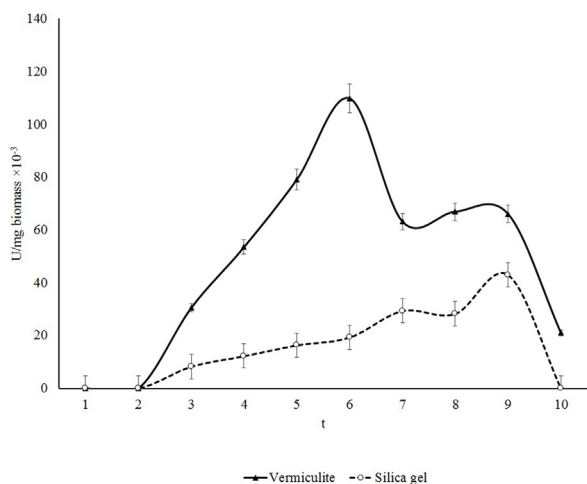


Fig. 5. Dynamics of proteolytic activity of micromycete *A. ustus 1*.

biomass with carrier and Petri dish; W2 is the weight of the carrier; W3 is the weight of Petri dish.

2.4. Determination and calculation of proteolytic activity

The proteinase activity of fungi was determined after preliminary separation of the biomass by filtration.

The general proteolytic activity and fibrinolysis were determined by Anson's modified method [14] using casein and bovine fibrin as substrates: 200 μ L of the culture liquid and 400 μ L of 1% suspension of the corresponding protein substrates prepared in 0.1 M Tris-HCl buffer (pH 8.2) were incubated 10 min at 37 $^{\circ}$ C. The reaction was stopped by 600 μ L of 10 % trichloroacetic acid. Measurements of the supernatant were taken at 275 nm after sample centrifugation. The activity was expressed in μ moles of tyrosine formed in 1 min in 1 mL of culture liquid.

Amidolytic activity was determined by the cleavage of the chromogenic peptide substrate D-valyl-L-leucyl-L-lisyl-p-nitroanilide (D-Val-Leu-Lys-pNA) as described in our earlier paper [7]. Incubation conditions were 10 min at 37 $^{\circ}$ C. The optical density was measured at 405 nm with an Eppendorf kinetics spectrophotometer. The amount of p-nitroaniline (μ mol) cleaved in 1 min was taken as one unit of activity.

Collagenolytic activity of the fungi was performed using the conventional azocoll technique with minor modifications [15,16]. Azocoll (Calbiochem, United States) in suspension (500 μ L, 2 mg/mL in 0.05 M Tris-HCl buffer, pH 8.2) was added to 250 μ L of the culture liquid filtrate; the mixture was incubated in a water bath for 15 min at 37 $^{\circ}$ C, and the reaction was stopped by adding 750 μ L of 10 % trichloroacetic acid. Specimens were centrifuged at 8500 g for 5 min, and the optical density of the supernatant was determined at 519 nm. The reaction was performed with constant stirring in a TS-100 thermal shaker (BioSan, Latvia). Collagenolytic activity was calculated using the calibration curve obtained by digesting exact amounts of azocoll with pronase E (1 mg/mL; Sigma-Aldrich, United States) to complete hydrolysis. The curve represented the relationship between absorption of the dye released by azocoll hydrolysis and its concentration. The level of collagenase activity was defined as the amount of azocoll (in μ g) degraded in 1 min.

Table 1
Activity of extracellular proteases produced by *A. ochraceus* L-1 and *A. ustus* 1 on SSF and SmF conditions.

Micromycete	Type of cultivation	Caseinolysis, U/mL	Fibrinolysis, U/mL	Amidolysis, U/mL
<i>A. ochraceus</i> L-1	SSF*	380.06	16,812	94.71
	SmF**	94.9	17.6	29.6
<i>A. ustus</i> 1	SSF*	551.6	224.4	108.51
	SmF**	25.1	22.1	39.0

* Growth on vermiculite, present study.

** Previous data [14].

The proteinase activity was calculated according to the formula $U_{SSF} = (A \times (V_m + V_b) \times C) / V_m$ [7], where A is the absorbance at suitable nm; V_m is the volume of the medium; V_b is the volume of the buffer added for elution, and C is the coefficient calculated from the calibration curve. For productivity calculation the obtained result were divided on amount mg of biomass.

The data were statistically processed using MS Excel 2013 and Statistica 7.0. The Mann–Whitney U test was used to compare the data; differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

Two strains of micromycetes, *Aspergillus ustus* 1 and *Aspergillus ochraceus* L-1 with high extracellular proteolytic activity were used in this study. The productivity of the mycelium of these strains was studied on the 5th day under SSF conditions using various inert carriers, based on own preliminary results and literature data [5, 18,19].

One of the disadvantages of SSF is excess heat, which can overheat the system and lead to enzyme denaturation. Therefore, in laboratory conditions, cultivation on small particles is often used. It is well known that small particles provide a large surface area for colonization while large particles limit the colonization area for micromycetes [10,20].

To select the best inert carrier for the production of extracellular proteinases SSF of micromycetes was carried out according to the laboratory scheme shown in Fig. 1. The sterile carrier was poured into the culture flasks, sterile nutrient medium was added and inoculated with a spore suspension of *Aspergillus*, as described in the procedure. During growth, nutrient uptake, proteolytic enzyme secretion, and mycelial differentiation occurred. At the end of the cultivation period, a buffer with a suitable pH (8.2) was added into the system and secreted enzymes were eluted. In the obtained enzyme extract proteolytic activity was determined.

Fig. 2 shows the productivity of aspergilli mycelium during their growth on various inert carriers. As can be seen from the obtained data, the proteolytic activity of both micromycetes was greatest with SSF on vermiculite (52.4 and 57.2 U/mg of biomass $\times 10^{-3}$ for *A. ochraceus* L-1 and *A. ustus* 1 respectively). In comparison with silica gel, the yield of proteinases on vermiculite was 3 times higher, compared with other carriers used - from 7 to 18 times. In both cases, the use of polyurethane foam and perlite as carriers in the solid-state fermentation system proved to be unpromising. Thus, the productivity of aspergilli mycelium on vermiculite was higher than on other carriers.

In this way, vermiculite can be considered as a suitable carrier for SSF of micromycetes - producers of proteinases.

Uninoculated vermiculite, vermiculite moistened with nutrient medium and vermiculite with aspergillus (*A. ustus* 1) grown on it are shown in Fig. 3. It is seen that the fungus fully develops in culture, as evidenced by dense growth and copious sporulation.

Strains of aspergilli, producers of proteinases with different types of activity were selected for this work. So, *A. ochraceus* L-1 is

considered as a producer of fibrinolytic (plasmin-like) proteinases, and *A. ustus* is a producer of extracellular collagenases [14].

To clarify the data obtained, the dynamics of extracellular proteases secretion during the growth of micromycetes *A. ustus* 1 and *A. ochraceus* L-1 under SSF conditions with vermiculite and silica gel (for comparison) as carriers, respectively were studied. Thus, the maximum value of the protease activity, produced by *A. ustus* 1 under SSF conditions with vermiculite, was obtained on the 6th day (109.8 U/mg of biomass $\times 10^{-3}$), the value of the protease activity, produced by micromycete *A. ochraceus* under similar conditions reached its maximum (170.5 U/mg $\times 10^{-3}$) only by the 8th day (Fig. 4, Fig. 5). Under the SSF condition, using silica gel as a carrier (Fig. 4, Fig. 5), the maximum value of the proteolytic activity of proteases, produced by *A. ustus* 1 was reached on the 9th day of cultivation and amounted to 42.9 U/mg of biomass $\times 10^{-3}$, and for *A. ochraceus* on the 5th day of cultivation (92.2 U/mg of biomass $\times 10^{-3}$). For both strains of micromycetes, the activities obtained under SSF conditions with vermiculite were higher than those using silica gel as a carrier: more than 2 times for *A. ustus* 1, and 1.5 times for *A. ochraceus* L-1.

The results obtained can be related to the physicochemical properties of the used vermiculite. During the experiments, fungal mycelium layering and dense entanglement of solid particles with hyphae were observed (Fig. 3), thereby the fungi increased their surface area of growth, and the properties of vermiculite allowed the secreted enzymes to be in an immobilized state. As a result of these factors, the productivity of *Aspergillus* mycelium is higher than when cultured on other carriers. The addition of excess buffer to the system made it easy to elute proteases from the carrier particles.

The activities of some other proteases, produced by *A. ochraceus* L-1 and *A. ustus* 1 under the same SSF conditions with vermiculite as a carrier were also studied. One way to compare activity in SmF and SSF is to calculate the activity per ml of culture medium added. In the case of laboratory SmF, as a rule, the growth and fermentation of micromycetes occur in 100 mL of nutrient medium, in the SSF on vermiculite given in this work the amount required is 4 times less. As can be seen in Table 1, SSF with vermiculite as a carrier is a suitable method for producing proteases with other activities: for example, under these conditions, both micromycetes produce caseinolytic proteases with high activity values – *A. ochraceus* L-1 (380.06 U/mL), *A. ustus* 1 (551.6 U/mL). Also, *A. ustus* 1 is capable of producing highly active fibrinolytic protease (224.4 U/mL), and *A. ochraceus* L-1 forms proteases with high amidolytic activity (94.7 U/mL) under these conditions. All types of activity were significantly higher than in the SmF of micromycetes [14].

Most studies on the formation of extracellular proteases by *Aspergillus* are carried out during their cultivation on agroindustrial wastes [19,21,22], which limits the possibility of scaling these processes and their industrial application. In cases with inert carriers, a high yield of fungal enzymes was previously noted during growth on polyurethane foam and perlite in comparison with the conditions of submerged cultivation [23–25]. In the present work, it was shown that during the development of

Aspergillus on vermiculite, the secretion of proteases is higher as compared to the conditions on the indicated carriers, which makes it promising for use in the conditions of SSF of *Aspergillus* - producers of proteolytic enzymes.

4. Conclusion

Currently, more and more attention is being paid to the production of proteolytic enzymes by SSF. By comparison with submerged cultivation, it was shown that in SSF producers, in particular *Aspergillus* of different species on different natural substrates and inert carriers, gave a yield several times (from 2 to 14 times) higher. Such data were obtained using polyurethane foam, silica gel, and amberlite as carriers [2,26]. In this work, it was shown that it is in principle possible to use vermiculite as a carrier in the SSF system to increase the yield of proteases. Studies have shown that the growth of *aspergillus* on vermiculite provides a higher yield of proteases in comparison with traditionally used carriers such as polyurethane foam, perlite and silica gel. The mycelium productivity of the studied fungi was higher not only in comparison with SmF, but also in comparison with other carriers under SSF conditions (from 3 to 18 times).

Thus, vermiculite is a suitable inert carrier for SSF of micromycetes - producers of extracellular proteases and a new effective way for protease production by some *Aspergillus* species under SSF with the usage of vermiculite as a carrier was developed.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

References

- [1] J. Barrios-González, Solid-state fermentation: physiology of solid medium, its molecular basis and applications, *Process. Biochem.* 47 (2) (2012) 175–185.
- [2] M. El-Bakry, J. Abraham, A. Cerda, R. Barrena, S. Ponsá, T. Gea, A. Sánchez, From wastes to high value added products: novel aspects of SSF in the production of enzymes, *Crit. Rev. Environ. Sci. Technol.* 45 (18) (2015) 1999–2042.
- [3] A. Pandey, Solid-state fermentation, *Biochem. Eng. J.* 13 (2–3) (2003) 81–84.
- [4] U. Hölker, M. Höfer, J. Lenz, Biotechnological advantages of laboratory-scale solid-state fermentation with fungi, *Appl. Microbiol. Biotechnol.* 64 (2) (2004) 175–186.
- [5] C. Sandhya, A. Sumantha, G. Szakacs, A. Pandey, Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation, *Process. Biochem.* 40 (8) (2005) 2689–2694.
- [6] L. Thomas, C. Larroche, A. Pandey, Current developments in solid-state fermentation, *Biochem. Eng. J.* 81 (2013) 146–161.
- [7] A.A. Osmolovskiy, V.G. Kreier, N.A. Baranova, A.V. Kurakov, N.S. Egorov, Production of Extracellular Proteinases - Protein C Activators of Blood Plasma - by the Micromycete *Aspergillus ochraceus* during Submerged and Solid-State Fermentation, *Appl. Biochem. Microbiol.* 49 (6) (2013) 581–586.
- [8] C. Webb, M.A. Manan, Design aspects of solid state fermentation as applied to microbial bioprocessing, *J. Appl. Biotechnol. Bioeng.* 4 (1) (2017) 511–532.
- [9] F. Boratyński, E. Szczepańska, A. Grudniewska, R. Gniłka, T. Olejniczak, Improving of hydrolases biosynthesis by solid-state fermentation of *Penicillium camemberti* on rapeseed cake, *Sci. Rep.* 8 (2018) 10157.
- [10] J. Mamo, M. Kangwa, H.M. Fernandez-Lahore, F. Assefa, Optimization of media composition and growth conditions for production of milk-clotting protease (MCP) from *Aspergillus oryzae* DRDFS13 under solid-state fermentation, *Braz. J. Microbiol.* 51 (2020) 571–584.
- [11] M.S. Valásková, G. Martynková, Vermiculite: structural properties and examples of the use // clay minerals in nature – their characterization, modification and application, E-Book. (2012), doi:<http://dx.doi.org/10.5772/51237>.
- [12] F. Balima, S. Le Floch, A. San-Miguel, L. Reinert, L. Duclaux, A.N. Nguyen, I. Daniel, A. Brület, L. Gremillard, V. Pischedda, Porosity evolution of expanded vermiculite under pressure: the effect of pre-compaction, *SN Applied Science* 1 (2019) Article 629.
- [13] A. Razaq, S. Shamsi, A. Ali, Q. Ali, M. Sajjad, A. Malik, M. Ashraf, Microbial proteases applications, *Front. Bioeng. Biotechnol.* 7 (110) (2019) 1–20.
- [14] A.A. Osmolovskiy, E.A. Popova, V.G. Kreyer, N.A. Baranova, N.S. Egorov, Fibrinolytic and collagenolytic activity of extracellular proteinases of the strains of micromycetes *Aspergillus ochraceus* L-1 and *Aspergillus ustus* 1, *Moscow Univ. Biol. Sci. Bull.* 71 (1) (2016) 62–66.
- [15] R. Chavira Jr., T.J. Burnett, J.H. Hageman, Assaying proteinases with azocoll, *Anal. Biochem.* 136 (2) (1984) 446–450.
- [16] T.S. Sharkova, A.V. Kurakov, A.A. Osmolovskiy, E.O. Matveeva, V.G. Kreyer, N.A. Baranova, N.S. Egorov, Screening of producers of proteinases with fibrinolytic and collagenolytic activities among micromycetes, *Microbiology* 84 (3) (2015) 359–364.
- [17] A.A. Osmolovskiy, N.A. Baranova, V.G. Kreier, A.V. Kurakov, N.S. Egorov, Solid-state and membrane-surface liquid cultures of micromycetes: specific features of their development and enzyme production (a review), *Appl. Biochem. Microbiol.* 50 (3) (2014) 219–227.
- [18] A.A. Osmolovskiy, E.S. Zvonareva, V.G. Kreyer, N.A. Baranova, N.S. Egorov, The effects of micromycete extracellular proteases of the *Aspergillus* genus on the proteins of the haemostatic system, *Russ. J. Bioorganic Chem.* 40 (6) (2014) 634–639.
- [19] G.E. Ortiz, D.E. Nosedá, M.C.P. Mora, M.N. Recupero, M. Blasco, E. Albertó, A comparative study of new *Aspergillus* strains for proteolytic enzymes production by solid state fermentation, *Enzyme Res.* (2016)3016149.
- [20] S.R. Couto, M.A. Sanroman, Application of solid-state fermentation to food industry – a review, *J. Food Eng.* 76 (3) (2006) 291–302.
- [21] N.S.A. Ahmed, S.G. Mohammed, A.A. El Hussein, M.A. El Siddig, Optimization of conditions for protease production from *Aspergillus niger* under solid state fermentation, *Global Advanced Research Journal of Microbiology* 6 (4) (2017) 21–29.
- [22] K.I. Chimbekujwo, M.I. Ja'afaru, O.M. Adeyemo, Purification, characterization and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2, *Scientific African* 8 (2020)e00398.
- [23] C. Hongzhang, W. Hui, Z. Aijun, L. Zuohu, Alkaline protease production by solid state fermentation on polyurethane foam, *Chem. Biochem. Eng. Q.* 20 (1) (2006) 93–97.
- [24] D. Heerd, S. Yegin, C. Tari, M. Fernandez-Lahore, Pectinase enzyme-complex production by *Aspergillus* spp. In solid-state fermentation: a comparative study, *Food Bioprod. Process.* 90 (2) (2012) 102–110.
- [25] S. Velasco-Lozano, T. Volke-Sepulveda, E. Favela-Torres, Lipases production by solid-state fermentation: the case of *rhizopus homothallicus* in perlite, in: G. Sandoval (Ed.), *Lipases and Phospholipases. Methods in Molecular Biology (Methods and Protocols)*, vol 861, Humana Press, 2012.
- [26] H. Chen, W. Hui, Z. Aijun, L. Zuohu, Alkaline protease production by solid state fermentation on polyurethane foam, *Chem. Biochem. Eng. Q.* 20 (1) (2006) 93–97.