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ALPHA-AMYLASE PRODUCTION FROM *Aspergillus oryzae M* BY SUBMERGED FERMENTATION

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The main goal of present study was implementation of the *Aspergillus oryzae M* strain improved technology using earlier developed method of microorganism selection. The 8 pure strains of *Aspergillus* fungi were screened for the production of extra cellular alpha-amylase using agar medium with starch as a substrate and incubated for 72h at 30 °C. Zone of clearance was observed for screening of the amylolytic fungi (in mm). *Aspergillus oryzae M* has demonstrated the highest zone of clearance. *Aspergillus oryzae M* was cultivated for 42 days in submerged conditions of growth using new method of fungal cultivation. This method based on immobilizing enzymes producers on solid career in submerged conditions of growth gives the way to improve quality of filtrates, which remain clear, does not require additional filtering and easily separated from the mycelium. Moreover, it allows to prolong the process of fungal cultivation and to maintain high enzymatic activity for a long period of time. Presented method allowed increasing alpha-amylase production from 321 U/ml (before immobilization) to 502 U/ml (after immobilization).

Key words: α-amylase, *Aspergillus oryzae*, submerged fermentation, immobilization.

Amylases are among the most important industrial enzymes. This group of enzymes occupies about 25% of the whole market of produced enzymes in the world [1, 2]. Despite the fact that among microorganisms that produce amylases there are bacteria, fungi, yeast and actinomycetes, in the recent period micromycetes got wide application, particularly *Aspergillus* fungi type thanks to the ease of its cultivation and high productivity [3, 4]. However, great drawback of industrial strains is their low activity, despite the fact that the chief requirement to enzymes is their high catalyst activity which is directly connected with the activity of the microorganism that produces this enzyme. Scientific research of the leading biochemical laboratories of the world are directed to the solution of this problem, i.e. obtaining of a highly active strain — superproducer of enzymes. The use of such highly active strain can reduce expenses on production of the enzyme, which will positively influence the price of the final product [5].

Until recently, the main instrument in obtaining such a highly active α-amylase strain was mutagenesis [6–9]. There are available data on increasing of *Aspergillus awamori* strain glucoamylase productivity obtained by using recombinant-DNA technology. As a result rising in the level of starch-splitting gene activator protein1, that was amyR gene-coded, led to 30% increasing in *Aspergillus awamori* strain productivity [10]. However, genetic engineering is long-lasting and requires for special complex techniques [11]. In this regards, the cells immobilization offer a multitude of advantages in enzymes production, such as high metabolic activity and strong resistance to toxic chemicals [12–15]. Immobilized microorganisms can be used for a long period of time with increased enzymatic activity [16, 17].

Traditionally used enzymatic agent processing technology applies the submerged culture method of micromycetes in periodic conditions. Under such conditions of micromycetes growth, their biomass forms

a shape of pellets, which during the culture process firms and becomes inaccessible for oxygen and nutrients. The biomass forms the maximum amount of the target product in 3-5 days only once. After that, this culture autolyzes and its enzyme activity decrease. A new method of cultivation of filamentous fungi has been developed by Bliyeva R.K., as well as devices and equipment for their cultivation [18]. Such devices and equipment prolong producers' cultivation period to 30–60 days and create the opportunity to obtain enzymes repeatedly in every 2–3 days of cultivation. This method is based on immobilization enzymes producers on solid career in submerged conditions of growth. Immobilization has a range of advantages: decreasing the price of the final product, absence of foreign substances, controlled process of enzyme-genesis, ability of various enzymes simultaneous production, etc. [19–21]. Design of proposed equipment gives the opportunity to increase the activity of immobilized cells culture filtrate comparing to free cells. In the present work α-amylase production using immobilized *Aspergillus* species was studied.

Materials and Methods

Screening for the alpha-amylase activity

The 8 pure strains of *Aspergillus* fungi (own collection) were screened for the production of extra cellular alpha-amylase using agar medium with starch using as a substrate (*Aspergillus awamori 16, Aspergillus awamori 1-8, Aspergillus awamori 22, Aspergillus oryzae M, Aspergillus oryzae 3-9-15, Aspergillus foetidus, Aspergillus niger П, Aspergillus niger 355*). *Aspergillus* fungi were maintained on Czapek medium. CZAPEK: $NaNO_3$ — 9.0 g, sucrose — 20.0 g; KH_2PO_4 — 1.0 g; $MgSO_4$ — 0.5 g; KCl — 0.5 g; $FeSO_4$ — 0.001 g; agar-agar — 20,0 g — per liter. The pure cultures were inoculated on starch agar medium and incubated for 72 h at room temperature. After obtained colonies of each plate iodine solution was layered on the agar plates and zone of clearance was observed for screening of the amylolytic fungi. A positive test is indicated by a clear, colorless zone around the growth. Only positive and better zone formed strain was taken for further study.

Enzyme production

For inoculum preparation, 25 ml of sterile distilled water was added to the 5-day-old culture grown on Czapek agar plate and scraped aseptically with inoculating loop. This suspension with spore concentration of $1.3 \cdot 10^7$ cells/ml, was used as inoculum for the fungal cultivation. Submerged fermentation was carried out in 750 ml Erlenmeyer flask by taking 100 ml of mineral salt medium (%): NH_4NO_3 — 0.5; KH_2PO_4 — 0.1; $MgSO_4$ — 0.05; KCl — 0.05; $FeSO_4$ — 0.001; maltose — 1.0; starch — 1.0. They were incubated at 30 °C on a rotary shaker (180 rpm) for 42 days. The growth medium was exchanged at 3-day intervals.

Alpha-amylase enzyme assay

The amylase activity was assayed by spectrohotometric measurement of a starch-iodine complex (State Standard of Russian Federation). The reaction mixture (15 ml) consisted of 10 ml of 1% (w/v) soluble starch and 0.5 ml appropriately diluted enzyme source in 25 ml of distilled water. After incubation at 30 °C temperature for 10 min the reaction was stopped by addition of iodine solution with 0.2 mol/dm^3 HCl. Then the enzymatic hydrolysis of starch was determined on spectrophotometer at 670 nm. One unit of the α-amylase activity was defined as the amount of enzyme that hydrolyses 1 g of starch for 1 hour in 30 °C, pH 4.7. The experiment was carried out in triplicates. The results were expressed as mean ± standard deviation using Excel 2010.

Results and Discussion

Screening for the alpha-amylase activity

During the initial screening of *Aspergillus* fungi for synthesis of alpha-amylase enzyme it was found that almost all studied strains grew on the starch medium. The clear zone formation concerns the ability of colonies with confirmed starch hydrolysis (Fig. 1).

Aspergillus oryzae M showed the highest clearance zone (29.3 mm) and was used for further studies (Table). Alpha-amylase activity of free cells of *Aspergillus oryzae M* after 3-days liquid cultivation was 321 U/ml.

Immobilization of Aspergillus oryzae M

A novel immobilization technique was developed by using the cheapest and most easily available cotton material as an immobilizing carrier for absorption of spores of *A. oryzae M*, which has been for the first time used for immobilization of microorganisms. Immobilized *A. oryzae M* on carrier is presented in Fig. 2.

Aspergillus oryzae M Control

Fig. 1. **Iodine clearing zone assay**

Starch hydrolysis test by *Aspergillus* fungi for alpha-amylase activity

NN	Strain	Clear zone, mm
1	*Aspergillus awamori* 16	11.5 ± 1.0
2	*Aspergillus awamori* 1-8	20.0 ± 0.9
3	*Aspergillus awamori* 22	14.3 ± 0.9
4	*Aspergillus oryzae M*	29.3 ± 2.8
5	*Aspergillus oryzae* 3-9-15	16.4 ± 1.9
6	*Aspergillus foetidus*	11.8 ± 1.0
7	*Aspergillus niger II*	16.7 ± 0.7
8	*Aspergillus niger* 355	12.1 ± 1.2

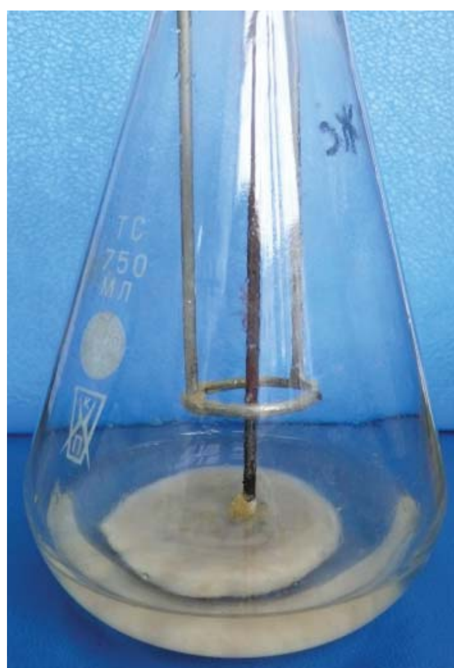

Fig. 2. **Immobilization of *Aspergillus oryzae M***

Cultivation period was extended to 42 days. Results show that immobilization procedure has significant effect both on growth and bioactivity of *A. oryzae M* when compared to periodic cultivation by free cells (Fig. 3). As shown on Fig. 3 enzymatic activity was enhanced significantly after 6 days of cultivation of immobilized cells and keeps the same value for 42 days of fungal cultivation. The immobilized system showed a significant stability of the enzyme biosynthesis. Maximum of the alpha-amylase production was obtained at 9 days interval. Enzyme activity ranged from 286 to 502 U/ml.

In contrast to immobilized cells cultivation by free cells has a limited development cycle. It forms greatest amount of alpha-amylase activity only on 3^{d} day. After that, *A. oryzae M* cells autolyze and its enzyme activity decrease (Fig. 3).

Thus, our presented method extends continuously without interruption for a period of 42 days. Frequency of obtainment of the desired product is gradually increasing. If the periodic cultivation by free cells allows to obtain target product (alpha-amylase enzyme) only once (on the 3^{d} day), during continuous cultivation by immobilized cells target product can be obtained every 2–3 days. In addition, presented immobilization procedure allows maintain high fungal enzymatic activity for a long period of time.

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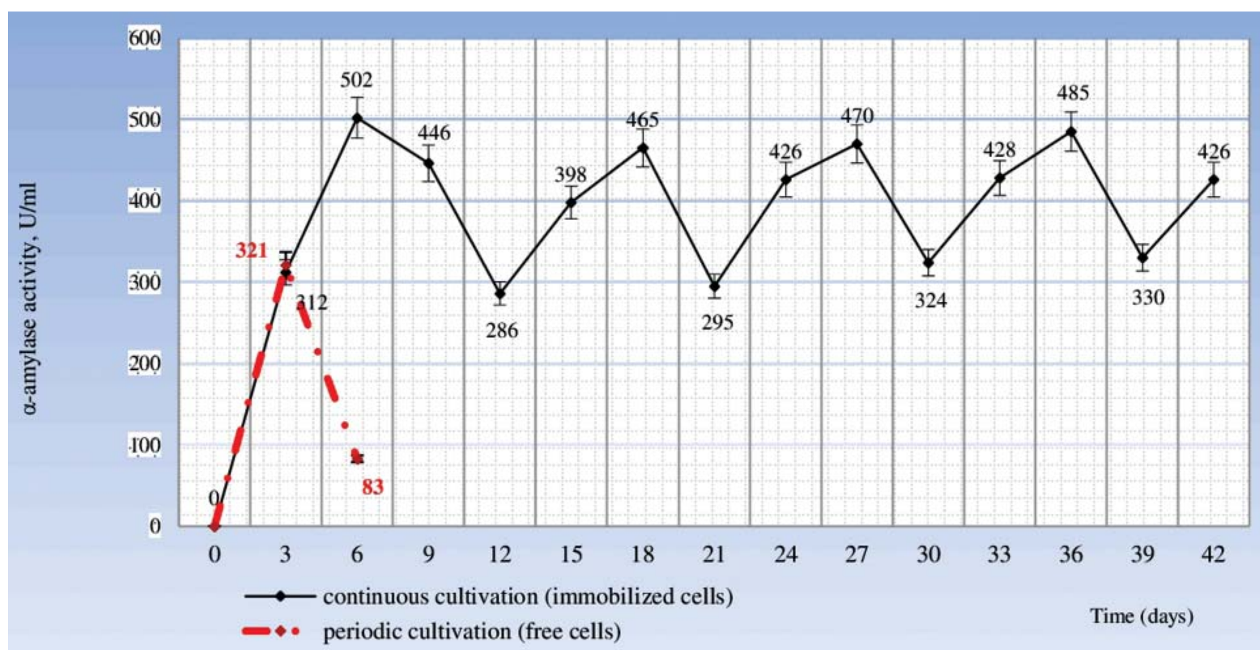

Fig. 3. **Alpha-amylase production by immobilized *Aspergillus oryzae M***

REFERENCES

1. *Rani G., Paresh G., Harapriya M., Vineet K. G., Bhavna C.* Microbial α-amylases: a biotechnological perspective. *Proc. Biochem.* 2013, V. 38, P. 1599–1616. doi: 10.1016/S0032-9592(03)00053-0.
2. *Lili Kandra.* α-Amylases of medical and industrial importance. *Journal of Molecular Structure: Theochem.* 2003, N 666, P. 487–498. doi: 10.1016/ j.theochem. 2003.08.073.
3. *David B. Finkelstein.* Improvement of enzyme production in *Aspergillus*. *Antonie van Leeuwenhoek J. Microbiol.* 1987, 53 (5), 349–352. doi: 10.1007/BF00400559.
4. *Ward O. P., Qin W. M., Dhanjoon J., Ye J., Singh A.* Physiology and Biotechnology of *Aspergillus*. *Adv. Appl. Microbiol.* 2005, V. 58, P. 1–75. doi: 10.1016/S0065-2164(05)58001-8.
5. *Vanek Z., Hosalek Z., Spizek J.* Overproduction of microbial products — Facts and ideas. *Biotechnol. Adv.* 1990, 8 (1), 1–27. doi: 10.1016/0734-9750(90)90003-T.
6. *Sidhu G. S., Sharma P., Chakrabarti T., Gupta J. K.* Strain improvement for the production of a thermostable α-amylase. *Enzyme and Microbial Technology.* 1997, 21 (7), 525–530. doi: 10.1016/S0141-0229(97)00055-0.
7. *Clark Ford.* Improving operating performance of glucoamylase by mutagenesis. *Cur. Opin. Biotechnol.* 1999, 10 (4), 353–357. doi: 10.1016/S0958-1669(99)80064-0.
8. *Parekh S.* Strain Improvement. *Encyclopedia of Microbiology, 3rd edn.* 2009, P. 286–298. doi:10.1016/B978-012373944-5.00169-3.
9. *Rowlands R. T.* Industrial strain improvement: Mutagenesis and random screening procedures. *Enz. Microb. Technol.* 1984, 6 (1), 3–10. doi: 0.1016/0141-0229(84)90070-X.
10. *Vinetsky Y. P., Rozhkov A. M., Sereda A. S., Tsurikova N. V., Nurtayev A. K., Semenov M. V., Zorov I. N., Sinicyn A. P.* Increase productivity strain *Aspergillus awamori* glucoamylase by a combination of radiation mutagenesis and plasmid transformation. *Appl. Biochem. Microbiol.* 2010, 46 (6), 685–692. doi: 10.1134/S0003683810060128.
11. *Sanjai Saxena.* Strategies of Strain Improvement of Industrial Microbes. *Appl. Microbiol.* 2015, P. 155–171. doi: 10.1007/978-81-322-2259-0-10.
12. *Cohen Y.* Biofiltration — The Treatment of Fluids by Microorganisms Immobilized into the Filter Bedding Material: a review. *Biores. Technol. Rev.* 2001, V. 77, P. 257–254. doi: 10.1016/S0960-8524(00)00074-2
13. *Liu H., Guo L., Liao S., Wang G.* Reutilization of immobilized fungus *Rhizopus sp. LG04* to reduce toxic chromate. *J. Appl. Microbiol.* 2012, N 112, P. 651–659. doi: 10.1111/j.1365-2672.2012.05257.x.
14. *Cai T., Chen L., Ren Q., Cai S., Zhang J.* The biodegradation pathway of triethylamine and its biodegradation by immobilized *Arthrobacter protophormiae* cells. *J. Hazard Mater.* 2001, V. 186, P. 59. doi: 10.1016/j. jhazmat.2010.10.007.
15. *Wang L., Ridgway D., Gu T., Moo-Young M.* Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. *Biotech. Adv.* 2005, V. 23, P. 115–129. doi: 10.1016/j. biotechadv.2004.11.001.
16. *Rhee S. K., Lee G. M., Lee S. T.* Influence of a supplementary carbon source on biodegradation of pyridine by freely suspended and immobilized *Pimelobacter* sp. *J. Appl. Microbiol. Biotechnol.* 1996, V. 44, P. 816–822. doi: 10.1007/BF00178624.
17. *Devi S., Sridhar P.* Production of cephamycin C in repeated batch operations from immobilized *Streptomyces clavuligerus*. *Proc. Biochem.* 2000, V. 36, P. 225–231. doi:10.1016/S0032-9592(00)00194-1.
18. *Blieva R. K.* An apparatus for the cultivation of microorganisms with filamentous structure. *Innovation Patent Kazakhstan № 27164*. June 25, 2013.
19. *Guisan J. M.* Immobilization of Enzymes and Cells (Third Edition). 2013, P. 115–119. doi: 10.1007/978-1-62703-550-7.
20. *Konstantin A., Lusta K. C., Whan S., Hee S. P., Dong S.* Immobilization of fungus *Aspergillus* sp. by a novel cryogel technique for production of extracellular hydrolytic enzymes. *Proc. Biochem.* 2000, 35 (10), 1177–1182. doi: 10.1016/S0032-9592(00)00158-8.
21. *Fiedurek J., Ilczuk Z., Lobarzewski J., Pleszczynska M.* Optimization of pectinolytic enzymes biosynthesis by immobilized mycelium of *Aspergillus niger 71*. *Zentralblatt f r Mikrobiologie.* 1992, 147 (1–2), 15–21. doi: 10.1016/S0232-4393(11)80357-4.

ПРОДУКУВАННЯ АЛЬФА-АМІЛАЗИ З *Aspergillus oryzae M* ЗА УМОВ ГЛИБИННОГО КУЛЬТИВУВАННЯ

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Метою дослідження було отримання високоактивного штаму-продуцента α-амілази на основі іммобілізації гриба *Aspergillus oryzae M* в умовах глибинного культивування за розробленим раніше методом селекції мікроорганізмів. Об'єктами досліджень слугували 8 штамів роду *Aspergillus* із колекції Інституту мікробіології та вірусології. Первинний відбір активної культури проводили якісним методом шляхом вимірювання діаметра зон гідролізу досліджуваними культурами субстрату (крохмалю) на третю добу інкубації за 30 °C (у мм). Найбільшу активність мав штам *Aspergillus oryzae M*. Проведено тривале культивування відібраного продуцента α-амілази *Aspergillus oryzae M* упродовж 42 діб. Активність α-амілази варіювала від 286 до 502 од/мл. Відзначено, що культуральна рідина прозора і не потребує додаткової фільтрації, легко відділяється від міцелію. Пропонований метод селекції уможливив підвищення активності альфа-амілази з 321 од/мл (до іммобілізації) до 502 од/мл (після іммобілізації), а також дав змогу зберегти активність культури протягом тривалого періоду часу.

Ключові слова: α-амілаза, *Aspergillus oryzae*; глибинне культивування, іммобілізація.

ПРОДУЦИРОВАНИЕ АЛЬФА-АМИЛАЗЫ ИЗ *Aspergillus oryzae M* В УСЛОВИЯХ ГЛУБИННОГО КУЛЬТИВИРОВАНИЯ

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Целью исследования было получение высокоактивного штамма-продуцента α-амилазы на основе иммобилизации гриба *Aspergillus oryzae M* в условиях глубинного культивирования по разработанному ранее методу селекции микроорганизмов. Объектами исследований служили 8 штаммов рода *Aspergillus* из коллекции Института микробиологии и вирусологии. Первичный отбор активной культуры проводили качественным методом путем измерения диаметра зон гидролиза исследуемыми культурами субстрата (крахмала) на третьи сутки инкубации при 30 °C (в мм). Наибольшую активность имел штамм *Aspergillus oryzae M*. Проведено длительное культивирование отобранного продуцента α-амилазы *Aspergillus oryzae M* в течение 42 суток. Активность α-амилазы варьировала от 286 до 502 ед/мл. Отмечено, что культуральная жидкость прозрачная и не требует дополнительной фильтрации, легко отделяется от мицелия. Предлагаемый метод селекции дал возможность повысить активность альфа-амилазы с 321 ед/мл (до иммобилизации) до 502 ед/мл (после иммобилизации), а также позволил сохранить активность культуры в течение длительного периода времени.

Ключевые слова: α-амилаза, *Aspergillus oryzae*, глубинное культивирование, иммобилизация.