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PARTIAL CHARACTERIZATION OF PENICILLIN ACYLASE FROM FUNGI *ASPERGILLUS FUMIGATUS* AND *MUCOR GRYSEOCIANUM*

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Preparation of Penicillin G acylase were obtained using two sources of fungus, *Aspergillus fumigatus* H/6.17.3 and *Mucor griseocyanum* H/55.1: The fungi were propagated in Skim milk medium at 30° as the sole nitrient sorce and D-Phenylglycine methyl ester as inducer. Dialyzed medium containing enzyme penicillin G acylase of *Mucor gryseocianum* and *Aspergillus fumigatus* was used. The phenylmethylsulfonyl fluoride was found to inhibit Penicillin G acylase activity of obtained preparations. The optimum pH range for the dialyzed preparations was pH 7–8 and pH 7.5–8.5 respectively and optimum temperature for maximal enzyme activity of both sources, was at 40°. Km value determined using the penicillin G as sustrate of enzymes of *Mucor gryseocianum* and *Aspergillus fumigatus* was 1.77×10^{-7} M and 1.46×10^{-7} M, respectively.

Potential of enzymes extraction from different microbial sources, that are able to catalyce many industrial processes offer great biotechnological possibilities. This give the possibility of choosing the most adequate industrial enzyme. An accurate selection of a given native enzyme may help to overcome a number of obstacles which hinder a massive implementation of enzyme derivatives as industrial catalysts [1].

Penicillin-G-acylase (PGA) (PG, EC-3.5.1.11) catalyses the hydrolysis of linear amide bond in penicillin molecules to produce the в-lactam nucleus, 6-aminopenicillanic acid (6-APA) and the corresponding carboxylic acid [2]. PG acylase is one of the most widely used enzymes at industrial scale for the production of semi-synthetic penicillins and cephalosporins, via 6-APA and 7-amino3-deacetoxy cephalosporanic acid (7-ADCA). The enzyme PG acylase catalyses the deacylation of penicillin G or Penicillin V under appropriate pH conditions. However it also has the ability to catalyse acylation of 6-APA under acid conditions [3]. The substrate specifity of penicillin acylase from different sorce has been investigated extensively. The enzyme that cleaves penicillin V is classified as type I, and type II enzyme have a high specifity for penicillin G [4, 5].

Penicillin acylase is produced by varios methods using a yeast, bacteria or mould. Microorganisms have been extensively screened for penicillin acylase production [5, 6]. Penicillin G acylase is, in general, produced in fermentative process and is obtained from either mutated or natural variant strains. The amount of enzyme produced varied acording to the composition of the medium and its constituents [7]. Several groups have reporte the production of penicillin G acylase from different microorganisms, and recencly fungy received more attention.

It has been demonstrated that fungi, produce and excrete to the middle penicillin acilasa with activity on the natural penicillins [4, 8]. Many efforts are being made to isolate new PGA of different sources, to extend its use at industrial level and to obtain less expensive biocatalysts with great especificity, activity, purity and stability in order to reduce the cost and finally to increase the production of 6-APA and semyntectic penicillins.

Based on a previous study on differents sources of enzymes, two fungal strains *Aspergillus fumigatus* and *Mucor gryseocianum*. were selected ant the present work was focused on the partial characterization of acylase obtained using these fungi.

MATERIALS AND METHODS

Material. Reagents 6-APA, p-dimethylamino benzaldehyde, penicillin G and phenylmethylsulfonyl fluoride and D-Phenylglycine methyl ester were obtained form Sigma. Methanol purchased from Merck and others reagents used in this study were of analytical grade.

Aspergillus fumigatus H/6.17.3 and *Mucor griseocyanum* H/55.1.1 were isolated from different natural sources and were characterized by the Institute of Sugar Cane Derivatives (ICIDCA). The strains were transferred on malt agar plates, incubated by 7 days to 30° C and stored to 4° C.

Fermentative essay. Cultures were grown aerobically under submerged conditions in 100 ml Earlenmeyer flasks, containing 20 ml of skim milk employed as medium. This

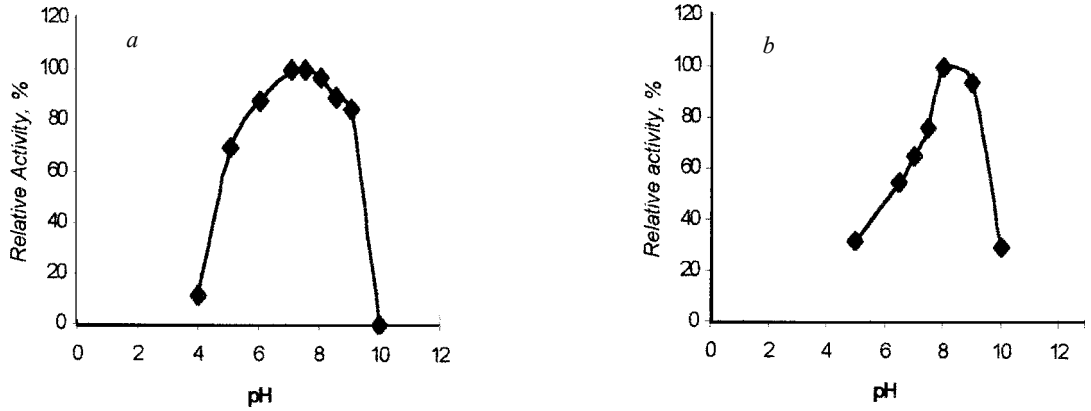

Fig. 1. Effect of the external solution pH on the activity Penicllin G acylace from: *a – A. fumigatus; b – M. gryseocianum* (40°C, 20mM Penicllin G)

medium container pure skim milk, without any additives. The phenilglycine methyl ester was employed as inducer. The inducer (0.5 g/l medium) was added aseptically after 24 h of fermentation. Fermentations were carriel out for 144 h at 30°C in incubation shaker (Eallemkamp, Germany). Cells were removed by filtration. The liquida fraction was dialyzed and the penicillin G acylase activity was determined.

Enzymatic assays. Penicillin G acylase activity was determined at 40°C with 20 mM penicillin G in 0.05 M phosphate buffer, pH 8.0. 6-APA formed as reaction product was estimated espectrophotometrically at 415 nm by the method using p-dimethylaminobenzaldehyde [9].

The optimum pH was determined for each samples by carrying out the reaction with a fixed amount of the enzyme at 40°C for 30 min. The system in each case was buffered with 0.05 M phosphate buffer (pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) and 0.05 M borate buffer (pH 8.0, 8.5, 9.0 and 10.0).

The optimum temperature was determined by checking the enzyme activity at different temperatures ranging from 20°C to 60°C. The reaction system in each case was buffered with 0.05 phosphate buffer (pH 8.0) and the reaction was allowed to procede for 30 min.

To meassure the activity-time relationship the reaction was performed at fixed time intervals within the range of 5–30 min at 40°C and buffered with 0.05 M phosphate buffer (pH 8.0) . The resultant activity was determined by quantifycation of 6-APA in the respective reaction mixture.

K_m and V_{max} was determinated by linewaver Burk plots and by assaying the rate of hydrolysis of penicillin G to 6-APA at different sustrate concentrations ranging from 1×10^{-6} M to 2×10^{-2} M. The reaction was carried out at 25°C for 30 min and buffered with 0.22 M phosphate buffer (pH 8.0). The enzyme activity of penicillin G acylase was confirmed and enzyme active site concentration was determined with phenylmethilsulfonyl fluoride (PMSF) titration using an earlier described method [10].

RESULTS AND DISCUSSION

It was shown that at 30°C, both the *Mucor gryseocianum* and *Aspergillus fumigatus* produces acceptable enzyme leves of penicillin acylase when grown on skim milk as the sole nitrient sorce and D-Phenylglycine methyl ester as inductor. D-Phenylglycine methyl ester was a good inducer of penicillin G acylase formation. As a very important resoult we observed that there no relation between celular growth and enzimatic leves [11], it is clear that the culture mediun imposes that favours the enzyme production [2].

Dialyzed form of the enzyme penicillin G acylase, from *Mucor gryseocianum* and *Aspergillus fumigatus* in our experiments. The optimum pH range for the dialyzed enzyme was pH 7–8 and pH 7.5–8.5 respectivily. However, showed a significant decrease in activity above pH 8.5 (Fig. 1). The results are similar in both strains and are in accordance with data obtained previusly with PGA from other sources. For example, the previos reports showed that optimum pH for PGA from *Penicillium chrysogenum* were pH 8–8.5 [12]. The decrease in activity abone pH 9.5 is due to alkaline degradation of pen G [12].

As is shown in Fig. 2, the optimum temperature for maximal enzyme activity was 40°C for the dilyzed form of PGA used in our experiments. However, for diferentes PGA obteined from differents sources, present optimum temperatura between 40°C and 60°C [7–12]. The obtained results suggets a thermostable nature of PGA, to demonstrate this, it is important to continue the studies in their semi-purified or purified form.

Both dialyzed preparations showed a linear activity – time relationship for 20 min and then reached a plateau (Fig. 3), that was used to calculate the initial rate and enzyme activity of preparations. An effective method stoichiometric by titration of penicillin acylase of *E. Coli* was developed and employed phenylmethylsulfonyl fluoride (PMSF) an extremely effective stoichiometric inhibidor, [10]. This method employd for determination of active enzyme concentrations in highly and partially purified preparations of penicillin acylase from differents strains [10]. In the present study, to demostrate that PG acylase was the enzyme responsable of hydrolitic activity, we determined the effect of different concentration of PMSF inhibidor on enzyme activity. PMSF showed to be an inhibidor of the acylase obtained of *Aspergillus fumigatus* and *Mucor gryseocianum* at micromolar concentrations. The inactivation

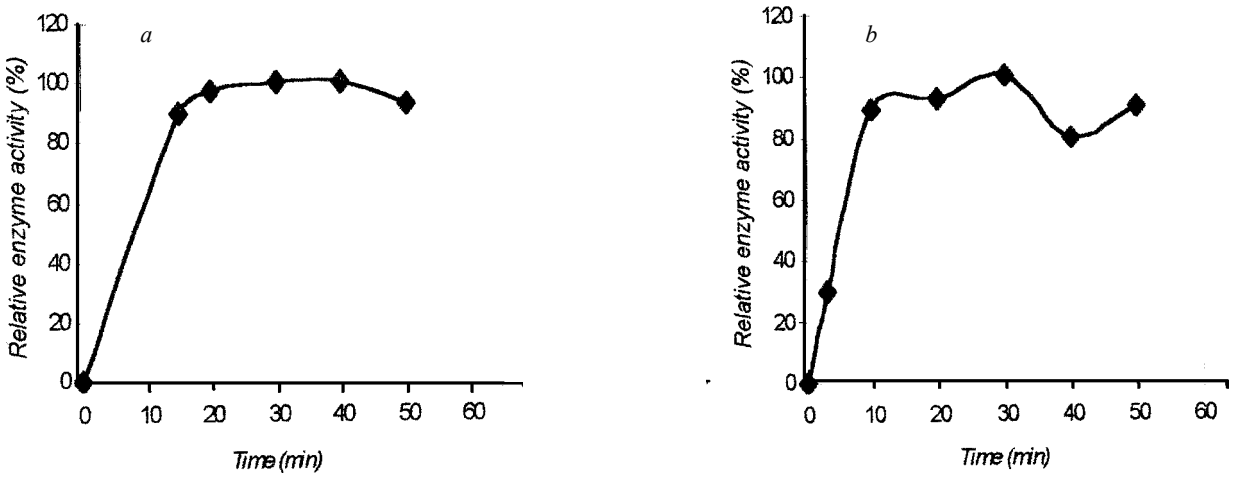

Fig. 2. Effect of temperature on the activity Penicllin G acylace from: *a – A. fumigatus; b – M. gryseocianum* (pH 8.0, 20 mM Penicllin G)

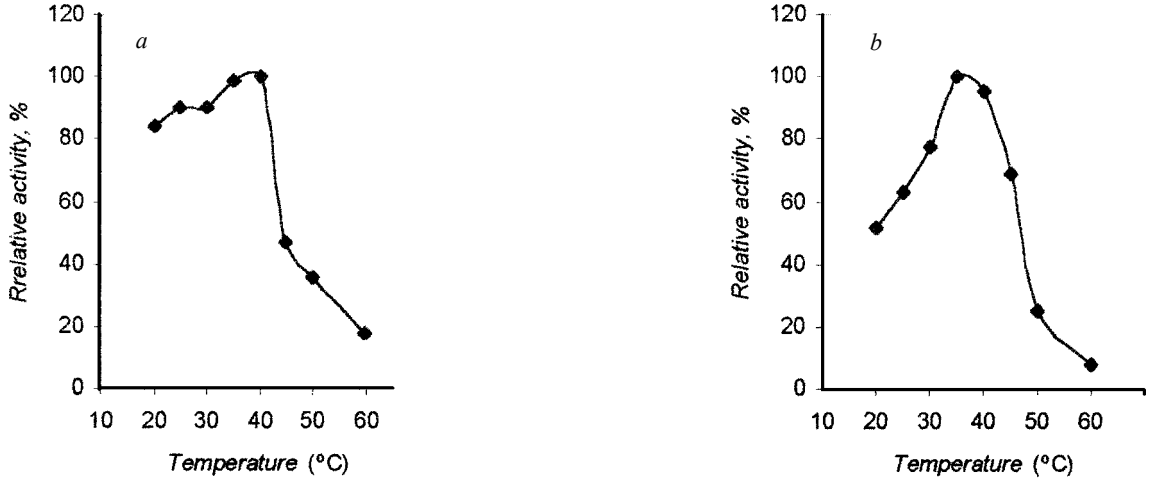

Fig. 3. Kinetic of conversion of Pen G (initial concentration 20 mM) to 6-APA catalized by Penicllin G acylace from: *a – A. fumigatus; b – M. gryseocianum* (pH 8.0; 40°C)

of penicillin G acylase take place in a few minutes (8 min), even at micromolar concentration of the this agent. Fig. 4, show that addition of equimolar concentration of PMSF inhibe the acylase activity at 25°C of temperatura, *Aspergillus fumigatus* and *Mucor gryseocianum* lost approximately 50% of its initial activity at 10 мМ and 18 мМ respectivily.

The hydrolysis of Penicillin G (PG) in the presence of penicillin G acylase obtained from various microorganisms species, is inhibited by high concentrations of sustrate, non-competitively by 6-APA and competitively by phenylacetic acid (PAA) which are the products of the hydrolysis reaction. The steady-state kinetic of the forward deacylation reaction of PG in the presence of PG acylase includes one substrato and two products [3, 10].

The kinetic of the enzymatic hydrolysis were studied under standard assay conditions using diffetent concentrations of sustrate (PG). The dependence of the inicial rate on the substrate concentration was measured and the experimental dates were determined by Lineweaver-Burk plots. Kinetics parameters were determined by computer regression analysis. The kinetic parameters determined for PG hydrolysis are show in Table. The Michaelis constants from *Mucor gryseocianum* and *Aspergillus fumigatus* (Table) indicate the affinity of enzyme to this substrato.

To our knowledge the current work is the first report of these two fungi, producing penicillin acylase in Skim milk as a medium. It is important to continued the study of these microorganisms with a large perspective on fungal biotechnology and industrial applications.

Parameters for the reaction by penicllin acylases from *A. fumigatus* and *Mucor gryseocianum*

Microorganism	K_m, M	V_{max}, M/min
Aspergillus fumigatus	1.46×10^{-07}	3.66×10^{-05}
Mucor Gryseocianum	1.77×10^{-07}	3.28×10^{-05}

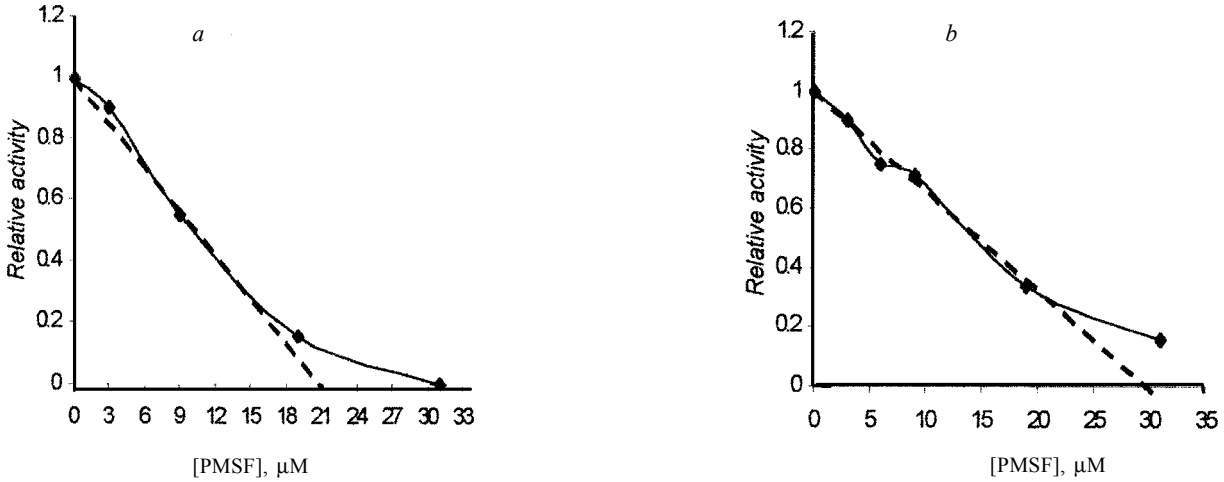

Fig. 4. Inhibition of Penicillin G acylace from activity by PMSF. Enzymatic preparation from: *a – A. fumigatus; b – M. gryseocianum* (pH 8.0; 25°C)

REFERENCES

1. *Alvaro G., Fernandez-Lafuente R., Rosell C.M., Blanco R.M., Garcia-Lopez J.L., Guisan J.M. //* Biotech. Letters. 1992. **14.** P. 285.
2. *Torres R., Ramyn F., de la Mata, Acebal C., Castillyn M.P.* //Appl. Microbiol. Biotech. 1999. **53.** P. 81.
3. *Kheirolomoom A., Ardjmand M., Fazelinia H., Zakeri A.* // Process Biochem. 2001. **26.** P. 1095.
4. *Anupama P., Harish K., Marwaha S.S., Kennedy J.F.* // Biotech. Advances. 2000. **18.** P. 289.
5. *Shewale J.G., Sivaraman H.* // Process Biochemistry. 1989. **24.** P. 146.
6. *Savidge T.A.* // Biotechnology of industrial antibiotics. N.Y., 1984. P. 171.
7. *Sudhakaran V.K., Shewale J.G.* // Hind Antibiotic Bull. 1995. **37.** P. 9.
8. *Vandamme E.J., Voetes J.P.* // Adv. Appl. Microbiol. 1974. **17.** P. 311.
9. *Balasingham K., Warburton D., Dunnill P., Lilly D.* // Biochim. Biophys. Acta.1972. **270.** P. 250.
10. *Svedas V., Guranda D., Lange L., Rantwijk F., Sheldon R.* // FEBS Letters.1997. **417.** P. 416.
11. *Martнnez H.J.L., Hernandez J.O., Dustet M.J.C., Sanchez C.O., Iliyna A.* // Biotecnology aplicada. 2002 (in printed).
12. *Meesschaert B., Espinosa S.G., Martin J.F.* // Genet. 1991. **56.** P. 1781.

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НЕКОТОРЫЕ СВОЙСТВА ПЕНИЦИЛЛИН АЦИЛАЗЫ ИЗ ГРИБОВ *ASPEGILLUS FUMIGATUS* AND *MUCOR GRYSEOCIANUM*

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Получены препараты пенициллин G ацилазы из грибов *Aspegillus fumigatus* H/6.17.3 and *Mucor gryseocyanum* H/55.1. Культуры выращивали на питательной среде на основе обезжиренного молока (коммерческое название *Skim milk*) при 30^0C, используя метиловый эфир D-фенилглицина в качестве индуктора. Получены ферментативные препараты путем диализа культуральной жидкости. Показано, что пенициллин ацилазная активность этих препаратов ингибируется фторидом фенилметилсульфонила. pH-оптимумы в реакции, катализируемой пенициллин ацилазой *M.gryseocyanum* и *A.fumigatus* были pH 7-8 и pH 7,5-8,5, соответственно. В обоих случаях наибольшая активность наблюдалась при 40°C. Значения K_m, определенные с использованием пенициллина G в качестве субстрата, составляли $1{,}77\cdot10^{-7}$ и $1{,}46\cdot10^{-7}$M соответственно.