Journal of Engineering and Applied Sciences 12 (7): 1920-1930, 2017
ISSN: 1816-949X

Obtaining and Genotyping of Fungal Producers of Proteolytic Enzymes

D. Oshanova, D. Bayanbek, S. Anuarbekova, B. Atabaeva, A. Abdraimova,
V. Remele and G. Ospankulova
Kazakh Scientific-Research, Institute of the Processing of Agricultural Products, Astana, Kazakhstan

Abstract: This research studied the ability to produce proteolytic enzymes in 27 fungal strains isolated from different crops. Proteolysis of casein and gelatin is studied. Proteolytic activity based on the modified method of Anson was evaluated. It is shown that fungi exhibiting high proteolytic activity during casein liquefaction may also show high activity and decomposition of gelatin. In spectrophotometric study showed the most active strains of *Penicillium bilaiae* 3-4,1 μM/mL, *Penicillium chrysogenum* 34/307-3,60 μM/mL, *Aspergillus fumigatus* 63/360-7,1 μM/mL. It also showed high numbers of casein liquefaction. Genotyping of six active cultures was made by direct method for determining the nucleotide sequence of ITS region (transcribed intergenic region) followed by determination of nucleotide identity with the sequences deposited in the international data base of Gene Bank. Identification was carried out by combination with phenotypic traits. Fungal culture in an amount of 27 will enrich the collection of the Institute.

Key words: Enzymes, grain, fungi, protease, proteolytic activity

INTRODUCTION

One of the main directions of biotechnology is producing by microorganisms essential amino acids, hormones, enzymes, vitamins, antibiotics, antiviral protein-interferon and increased use of microorganisms in the food industry. Enzymes are very important (Pandey *et al*., 2000).

Enzymes of microbial origin gradually replaced vegetable and animal enzymes because their production was more profitable. Most gross amounts of enzymes in particular hydrolytic are produced by culturing the bacilli, lactobacilli, fungi of the genus *Aspergillus*, *Penicillium*, *Trichoderma* and others (Sanni *et al*., 2002; Huch *et al*., 2008; Ogbonnaya and Odiase, 2012; Pascoal *et al*., 2011; Do *et al*., 2013; Li *et al*., 2013; Patil *et al*., 2013; De Souza *et al*., 2015).

The use of microbial enzymes in plant growing, food and light industry can significantly intensify the process, increase the yield and improve the quality of final products. The main share of production comes from proteolytic enzymes.

Microbial proteases are one of the most important hydrolytic enzymes (De Souza *et al*., 2015). Proteases from microorganisms have attracted much attention in the last decade due to their biotechnological potential in various industrial processes and in connection with the technical and economic benefits (De Souza *et al*., 2015; Kuddus and Ramteke, 2012). This group is one of the largest groups of industrial enzymes; it is about 60% of the total sales of the enzyme in the world where two-thirds is proteolytic enzymes (Dias *et al*., 2008; Rodarte *et al*., 2011).

The first scientific discoveries related to the processes of proteolysis have long been known. Moreover, proteolysis borders are constantly expanding, it involves a series of problems and issues which initially seemed to have no relation to it (De Souza *et al*., 2015). And above all, this is new technologies in the development of the food industry (Pandey *et al*., 2000; Sanni *et al*., 2002; Huch *et al*., 2008). These technologies are used as meat softeners, maturation agents for meat and fish. In conducting the weak proteolysis using specific sets of enzymes a slight change of meat structure is occured but the quality is getting better, meat becomes much softer (Kumari *et al*., 2012; Tomar *et al*., 2008; Kumar and Takagi, 1999). The activity of enzymes is particularly important in the connective tissue proteins. In this case, it is possible to make better use of all parts of the carcass. The proteases allow using even high-protein flour in the production of confectionery products with a long shelf life (Rao *et al*., 1998). The quality of the final product increases significantly. Coagulation of milk by the action of proteinase rennet of calves and lambs chymosine is an important process step in the cheese making (Rao *et al*., 1998). Other proteases also coagulate milk caseinogen but with lower yield and lower strength of casein clot. Thus, to achieve the same coagulation ability it is required 10 times more pepsin than chymosine

Corresponding Author: Anuarbekova, Kazakh Scientific-Research, Institute of the Processing of Agricultural Products, Astana, Kazakhstan

and fungal proteases or proteases from *Bacillus subtilis* in 25 and 80 times as much, respectively. During brewing by proteases in the malting process, wort production (during mashing) to accelerate these processes, improving the quality of beer, its flavor, aroma and firmness, using malt of poor quality and unmalted materials it is necessary to add proteolytic agents.

Proteases are also used to dissolve the protein turbidity in beer which often comes as a residuum during storage in the cold. Hydrolyzing protein components of green beer, proteinase can improve its filterability, increase transparency and colloidal stability. They are added in the green beer on postfermentation stage. Proteinases preparations from fungi such as amiloprotoorizin, protoorizin contain complex proteinases and peptidases (>5 proteolytic enzymes) which hydrolyse proteins to small peptides and amino acids assimilated by the yeast cell. The use of such preparations can remove the colloidal protein formation and provide yeast with nitrogen nutrition. By increasing the fermentation activity of yeast (20-25%) the process of alcoholic fermentation is intensified (30-40%), the ethanol yield is increased (Netrusov *et al*., 2005). Fungal Protease has clinical significance, improving digestibility and sensory quality of food products, contributing the susceptibility to allergenic compounds (Tavano, 2013).

Proteolytic enzymes produced by fungi of the genus *Aspergillus*, *Penicillium*, bacteria of the genus *Bacillus*, yeast genus *Saccharomyces*. Proteases obtained as a product of the biosynthetic activities of fungi or bacteria (De Souza *et al*., 2015; Anitha and Palanivelu, 2013). However, more preferably the use of fungi than bacteria, because they synthesize a complex set of proteases that can be easily changed, growing on cheap substrates in combination with high-performance (De Souza *et al*., 2015; Germano *et al*., 2003). In this regard, the selection of new fungal strains for protease remains urgent.

MATERIALS AND METHODS

Isolation and identification of microbial cultures: Isolation of the microorganisms was carried out in accordance with methods adopted in microbiology (Germano *et al*., 2003). Isolation of pure culture generally involves three stages: obtaining enrichment culture; isolation of a pure culture; determining the purity of isolated cultures.

Biomaterial is introduced in the nutritious fluids for obtaining enrichment culture. Biomaterials are wheat grains, corn, barley, rapeseed, rice, oats, soybeans and bran. The 5 g of the grain mass was placed in a beaker with 50 mL sterile tap water. The beaker was shaken in circular rotational motions for 10 min. Inoculation was made from extract on the surface of a dense medium. After the inoculation the dishes were placed in an incubator with their lids downwards, so that condensated water that had been forming on the lid during solidification of the agar would not prevent obtaining of the isolated colonies. Dishes are incubated for 5-7 days depending on the growth rate of the microorganisms. The grown fungal colonies are sifted with loop on the surface of dense medium in the beaker.

The purity of isolated cultures of microorganisms should be thoroughly checked. During visual inspection the growth of microorganisms according to the line on the sloping surface is checked. If growth is not uniform to the line, the culture is contaminated.

Pure culture of microorganisms is required to control aunder a microscope and check by inoculation on the nutritional medium. For the isolation and cultivation of fungi the czapek medium was used. Identification was carried out by the determinant formed by cultural-morphological and physiological features of fungi (http://www.agroatlas.spb.ru; http://www.cbs.knaw.nl/databases; Samson and Pitt, 2000).

Proteolysis of casein: Sterile skimmed milk was added to 15 mL of sterile water bath of molten agar Czapek medium 3 mL of. The contents of the beakers were mixed thoroughly and poured into a petri dish. Studied bacteria cultures were inoculated by plaques (5-7 days at 26°C).

Cultures producing proteolytic enzyme cause peptonization of milk protein (casein) whereby around the colonies transparent zones clearly stand out from the general background of milky turbid medium. The diameter of the hydrolysis zone is measured.

Proteolysis of gelatin: To 100 mL of Czapek bouillon 15 g of gelatin was added, leaving 30 min for swelling and then the mixture was heated on a water bath until its dissolution and the resulting medium was poured to 10 mL. Sterilized by 0.5 atm for 15 min. Inoculation was produced by an injection in the medium. Duration of the cultivation is 7-10 days at room temperature. Liquefaction of gelatin or its absence is noted visually. It is ncessasry to indicate the amount and form of thinning, layering, funnel, sacculate etc. after liquefaction of gelatin.

Protease activity: Modified Anson method was used to determine protease activity. The method is based on determining the tyrosine (or tyrosine peptides) that are

released during the hydrolysis of the protein standard solution (casein) by proteases (peptidhydrolases). Number of formed amino acids (tyrosine) was measured with a spectrophotometer.

One unit of proteolytic activity of the enzyme's ability is converting for 1 min at 30°C in sodium caseinate in nonsettleable state trichloroacetic acid in an amount corresponding to 1 micromol of tyrosine. The proteolytic activity is expressed in number of indicated units for 1 g of the test preparation.

As a substrate 2% sodium caseinate was used. Take three beakers (one check beaker and two pilot beakers). The pilot beakers filled with 2 cm of sustrate and placed in ultraincubator at 30°C for 10 min, then each beaker is filled with 2 cm of fungus culture liquid (previously treated at 30°C for 3-4 min), the beakers are shaked and left for hydrolysis for 10 min at 30°C. To stop reaction after 10 min 4 cm of 0.3 M solution of trichloroacetic acid is added to both beakers. Then th mixture is rapidly stirred and to ensure complete settlement hold the beaker at 30°C for an additional 20 min. Then the mixture is filtered into the dry beakers and the absorbancy determined at a wavelength of 656-670 nm.

Genotyping microorganisms of active cultures: Genotyping of active cultures carried out in several stages:

- Extraction of fungi DNA (Clayton *et al*., 1995)
- Amplification of the ITS region
- Determination of nucleotide sequence (Werle *et al*., 1994; Zhang *et al*., 2002; Clarridge, 2004; Kumar *et al*., 2004)
- Nucleotide sequence analysis was carried out with the construction of a phylogenetic tree from the sequences deposited in GeneBank international data base

RESULTS AND DISCUSSION

Proteases of microbial origin are of great biotechnological importance. The search for new producers of proteases is a selection of the proteolytic activity among newly selected or mutant strains of microorganisms. Proteolytic enzymes catalyze the breakdown of proteins to poly and oligopeptides, extracellular protease activity is determined using substrate such as gelatin, casein or other proteins. In our experiments on the number of liquefied gelatin and breakdown casein we judged on the relative activity of the proteolytic enzymes of different mushrooms.

Objects of the study: The studied fungi were isolated from a variety of cereals (wheat, corn, barley, rapeseed, rice, oats, soybeans, bran). The 27 fungal cultures were isolated from the grains on Czapek medium. Identification of genus was carried out based on the culture-morphological, physiological and biochemical characteristics. Fungi represented by the genus *Penicillium* sp., *Aspergillus* sp., Talaromyces *sp.* (Table 1).

Table 1: Fungi cultures used in work

Culture names	Source of isolation
Penicillium sp. 37/272	Wheat
Penicillium sp. 65/375	Wheat
Penicillium sp. 67/429	Wheat
Penicillium sp. 69/424	Barley
Penicillium sp. 192	Canola
Penicillium sp. 263	Rice
Penicillium sp. 66	Wheat
Penicillium sp. 62	Wheat
Penicillium sp. 3	Wheat
Penicillium sp. 195	Oat
Penicillium sp. 36/261	Wheat
Penicillium sp. 10	Bran
Penicillium sp. 35/263	Wheat
Penicillium sp. 64/373	Wheat
Penicillium sp. 66/381	Wheat
Penicillium sp. 79/494	Corn
Penicillium griseofulvum 282	Wheat
Penicillium sp. 83/571	Rice
Penicillium chrysogenum 34/307	Corn
Penicillium sp. 77/574	Soy
Penicillium sp. 12	Wheat
Aspergillus fumigatus 63/360	Corn
Aspergillus awamory 183/1	Soy
Aspergillus terreus 54/375	Wheat
Aspergillus flavus 70/404	Wheat
Aspergillus ochraceus 57	Corn
Talaromyces flavus 256	Corn

Cultivation of isolated fungi was carried out on a liquid Czapek medium and the ability of fungi to discharge a particular enzyme. This was evaluated by extracellular activity through which fungi penetrate into the interior of the plant tissue. Incubation was carried out at 26°C for 7 days and at the end of fermentation, the enzyme activity was determined.

Examination of proteolytic activity: The proteolytic activity of the cultures in working collection was determined by the number of liquefied gelatin on the 7th days of cultivation (Table 2). If gelatin liquefies the intensity is indicated (Netrusov *et al*., 2005).

Amount of liquefied gelatin in the beaker on the 7th day of fungi strains cultivation ranged generally from 4.5-70 mm. The lowest activity of proteolytic enzymes (4.5 mm) was observed in the strain *Penicillium* sp. 62. The next lowest figure observed in *Penicillium* sp. 36/261-5.5 mm. Indicators of other fungal cultures varies in the range of 9.5-31 mm. Two *Penicillium* strains have a high rate-*Penicillium* sp. 3 and *Penicillium chrysogenum* 34/307: 52.5 and 57.5 mm, respectively. Out of five strains of *Aspergillus* fungi three showed weak

Table 2: The proteolytic activity of isolated microscopic fungi

Culture name	Casein hydrolysis zone (mm)	Column of gelatin liquefaction (mm)	Intensity of the gelatin liquefaction (+/-)
Penicillium sp. 37/272	-	-	----
Penicillium sp. 65/375	-	17.5	++--
Penicillium sp. 67/429	-	10.5	+---
Penicillium sp. 69/424	4.5	-	----
Penicillium sp. 192	4.5	15.0	+---
Penicillium sp. 263	8.5	16.0	+---
Penicillium sp. 66	3.0	-	----
Penicillium sp. 62	1.0	4.5	+---
Penicillium sp. 3	10.5	52.5	+++-
Penicillium sp. 195	3.0	27.5	++--
Penicillium sp. 36/261	8.5	5.5	+---
Penicillium sp. 10	5.5	12.5	+---
Penicillium sp. 35/263	-	-	----
Penicillium sp. 64/373	8.5	29.0	++--
Penicillium sp. 66/381	7.5	9.5	+---
Penicillium sp. 79/494	5.0	10.5	+---
Penicillium griseofulvum 282	6.5	31.0	++--
Penicillium sp. 83/571	5.5	17.5	++--
Pen. chrysogenum 34/307	10.0	57.5	+++-
Penicillium sp. 77/574	6.5	16.0	+---
Penicillium sp. 12	3.5	9.5	+---
Aspergillus fumigatus 63/360	3.0	70.0	++++
Aspergillus awamory 183/1	18.0	31.0	++--
Aspergillus terreus 54/375	10.0	10.0	+---
Aspergillus flavus 70/404	10.0	10.0	+---
Aspergillus ochraceus 57	10.0	11.0	+---
Talaromyces flavus 256	15.0	20.0	++--

proteolytic activity (10 and 11 mm). The exception was the strain of *Aspergillus fumigatus* 63/360 a column of gelatin reached 70 mm which corresponds to the highest result. L.N. Kursanov noted that many types of *Aspergillus* fungi are used as a good source of protein nitrogen supply which in this case is subjected to hydrolysis, for others there are no hydrolysis and protein. This difference can be used in some cases as a systematic sign. Strain *Talaromyces flavus* 256 was less active. Column of gelatin liquefaction reached 20 mm. Four strains do not possess the ability to liquefy gelatin: *Penicillium* sp. 37/272, *Penicillium* sp. 69/424, *Penicillium* sp. 66, *Penicillium* sp. 35/263.

The intensity of the liquefaction was the percentage of the liquefaction respectively from ---- to ++++. Liquefaction was funnelar for all cultures. Thus, the majority of the fungal cultures in medium were actively discharging proteolytic enzymes. Column of gelatin was liquified in the most cases. Changing medium pH to 9 shows a high enzyme activity of these strains. Among the 27 studied cultures of microscopic fungi the following strains rapidly decomposed gelatin *Penicillium* sp. 3, *Aspergillus fumigatus* 63/360, *Penicillium chrysogenum* 34/307 column liquefaction was within 50-70 mm. However, these 3 highly active strains showed different ability of casein substrate decomposition: *Penicillium* sp. 3-10, 5, *A. fumigatus* 63/360-3, *P. chrysogenum* 34/307-10 mm (Table 2 and Fig. 1).

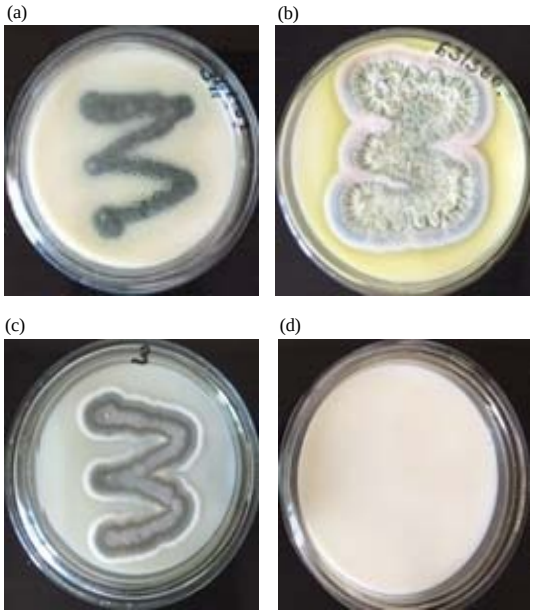

Fig. 1: Hydrolysis of casein by microscopic fungi which have a high rate of gelatin hydrolysis: a) *Penicillium chrysogenum* 34/307; b) *Aspergillus fumigatus* 63/360; c) *Penicillium* sp. 3 and d) Control

Hence, it is not an indication that the decomposition of one protein leads to the decomposition of other active protein. Four strains do not decompose milk protein, two strains are not decompose gelatin: *Penicillium* sp. 37/272 and *Penicillium* sp. 35/263.

Fungi give active indicators within 3-18 mm. The most active are *Aspergillus awamory* 183/1-18 mm and 256 *Talaromyces flavus*-15 mm. One strain of *Aspergillus* gives 3 mm of activity, the rest is 10 mm. Among the fungi of *Penicillium* genus greatest activity was shown by *Penicillium* sp. 3 and *Penicillium chrysogenum* 34/307, 10.5 mm and 10 mm, respectively. Subsequently the modified Anson method was used to determine protease activity. As a substrate 2% sodium caseinate was used. Spectrometric examination results are shown in Table 3.

As a result of this work it was found out that the fungal strains studied to some extent exhibit enzymatic activity but some fungi have little or no activity of the enzyme, i.e., fungi differed in terms of extracellular enzyme activity quantity.

Table 3 shows that the greatest activity was shown by proteolytic strains of *Penicillium* sp. 3-4.1 μM/mL, *Penicillium chrysogenum* 34/307-3.60 μM/mL, *Aspergillus fumigatus* 63/360-7.1 μM/mL. These objects had a good performance during evaluation of

Table 3: The proteolytic activity according to Anson

Culture name (2)	The amount of amino acids formed (μM/mL)
Penicillium sp. 37/272	0.33
Penicillium sp. 65/375	2.05
Penicillium sp. 67/429	0.71
Penicillium sp. 69/424	0.20
Penicillium sp. 192	0.82
Penicillium sp. 263	0.74
Penicillium sp. 66	0.56
Penicillium sp. 62	0.90
Penicillium sp. 3	4.10
Penicillium sp. 195	2.24
Penicillium sp. 36/261	1.66
Penicillium sp. 10	1.89
Penicillium sp. 35/263	0.11
Penicillium sp. 64/373	2.57
Penicillium sp. 66/381	1.11
Penicillium sp. 79/494	0.60
Penicillium griseofulvum 282	1.09
Penicillium sp. 83/571	1.07
Penicillium chrysogenum 34/307	3.60
Penicillium sp. 77/574	0.55
Penicillium sp. 12	0.51
Aspergillus fumigatus 63/360	7.10
Aspergillus awamory 183/1	3.00
Aspergillus terreus 54/375	0.60
Aspergillus flavus 70/404	1.00
Aspergillus ochraceus 57	1.10
Talaromyces flavus 256	2.80

casein and gelatin liquefaction. *Penicillium* sp. 3: liquefaction of gelatin -52.5 mm (+++ -) liquefaction of casein -10.5 mm; *Penicillium chrysogenum* 34/307: 57.5 mm (+++ -) and 10 mm; *Aspergillus fumigatus* 63/360: 70 mm (++++) and 3 mm.

The following fungi were less active *Penicillium* sp. 64/373 (2.57 μM/mL), *Aspergillus awamory* 183/1 (3.00 μM/mL), *Talaromyces flavus* 256 (2.8 μM/mL), respectively, they had good performance (Table 2).

However, this is not regularity as for example, strains of *Penicillium* sp. 263, *P. griseofulvum* 282, *A. terreus* 54/375, *A. flavus* 70/404, *A. ochraceus* 57 showed a high proteolytic activity during hydrolysis but have a low rate during spectrometric study (Table 2 and 3). Work on the further study of the strains properties with high proteolytic activity will be continued to form a collection of industrial and applied strains.

We screened the 27 studied fungal cultures and selected the most active: *P. chrysogenum* 34/307, *Penicillium* sp. 3, *P. griseofulvum* 282, *A. fumigatus* 63/360, *A. awamory* 183/1, *T. flavus* 256. Further on genotyping of these fungi is presented.

Genotyping of fungal cultures: Identification of 6 fungi strains has been carried out by direct nucleotide sequence of ITS region (transcribed intergenic region). This followed by determination of nucleotide identity with the sequences deposited in the international data base of Gene Bank. Designation of study objects is given in Table 4.

Table 4: Name of samples for genotyping of fungal cultures

Designations	Culture name
1	34/307
2	Pg3
3	63/360
4	282
5	183/1
6	256

Table 5: DNA concentration

Samples	Culture names	Concentration of DNA ng/uL	A260	A280	260/280
1	34/307	21.890	0.438	0.213	2.06
2	Pg3	19.840	0.397	0.293	1.35
3	63/360	19.500	0.390	0.207	1.89
4	282	5.140	0.103	0.033	3.13
5	183/1	6.430	0.129	0.065	1.99
6	256	20.450	0.406	0.210	2.05

Extraction of fungi DNA (Clayton *et al.*, 1995): For the extraction of DNA the buffer was used that consists of 100 mM Tris-HCL, pH 8,0, 1,4 M NaCL, 20 mM EDTA, 2% CTAB, Proteinase K 100 μg/mL. The cultures were centrifuged, supernatant was removed and residuum was transferred to a sterile mortar. Liquid nitrogen was added and milled to a powdered state. Then 100 mcl of the resulting suspension was transferred to a sterile 1.5 mL beaker. Then, 500 MCL of the corresponding buffer was added. The beaker was incubated for 18 h. Further on the purification was performed by phenol/chloroform method, for this purpose 750 MCL of chloroform/isoamyl alcohol (24/1) was added, thoroughly stirred and centrifuged at 12000 rev/min for 10 min. The aqueous phase was transferred to a new beaker and purified by phenol/chloroform/isoamyl alcohol (24/24/1). After centrifugation, the aqueous phase was transferred to a new clean beaker and DNA was precipitated at 0.6 volumes of isopropyl alcohol. Centrifuged at 12000 rev/min for 10 min, the DNA residuum was washed once with 70% ethanol, followed by centrifugation and removal of the aqueous phase. Then, the residuum was dried in air for 15 min. DNA samples were dissolved in 100 MCL of TE buffer once and stored at -20°C. DNA concentration was measured spectrophotometrically using NanoDrop spectrophotometer at a wavelength of 260 nm (Table 5).

Amplification of ITS region: PCR was carried out with the following primers ITS 5 5'-ggaagtaaaagtcgtaacaagg-3' and ITS 4 5'- cctccgcttattgatatgc-3' in total volume of 30 mcl. PCR mixture contained 10 ng of DNA, 1 unit Taq DNA Polymerase (Amplechem), 0.2 mM of each dNTP, 10×NH4 of buffer (Amplechem), 10 pmol of each primer. PCR amplification program included long denaturation at 94°C for 5 min; 30 cycles at 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min; final elongation at 72°C for 7 min, PCR

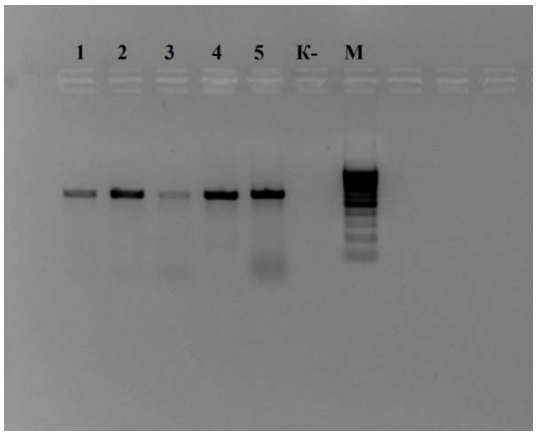

Fig. 2: Electrophoretic slide of ITS region of amplified PCR products; (1-5) samples, numbered according to numerical order; (M) marker of molecular weight (Fermentas) (100-10000 bp from 100-1000 with pace 100 bp) (κ-) negative control sample

was performed with the program using amplifier 2 DNA Engine Tetrad Cycler PTC-0240 (Bio-Rad). PCR amplification results are shown in Fig. 2. As shown in Fig. 2 a specific band is present in all wells indicating the presence of PCR product.

Determination of nucleotide sequence: The purification of PCR products from free primers was carried out using an enzymatic method, Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) (Werle *et al*., 1994).

Sequencing reaction was performed using BigDye®Terminator v3.1 Cycle Sequencing kit (Applide Biosystems) according to manufacturer's instructions, followed by separation of the fragments on an automated Genetic Analyzer 3730xl DNA Analyzer (Applide Biosystems).

Nucleotide sequence analysis: The nucleotide sequences were analyzed and combined into a common sequence in the Software SeqMan (DNA Star). Then terminal fragments (nucleic acid sequences of primers, fragments with a low quality level) have been removed. The sequences were identified by BLAST algorithm in GeneBank. The results are shown in Table 6.

In addition, phylogenetic trees were constructed with the sequences deposited in international data base GeneBank. Mega 5 Software was used to construct phylogenetic trees. We used ClustalW algorithm to align the nucleotide sequences, construction of a tree was performed by using the method of the nearest neighbor attachment (Neiighbor-Joining NJ).

The published data (Zhang *et al*., 2002; Clarridge, 2004; Kumar *et al*., 2004) indicates the presence of error in international banks of nucleotide sequences GeneBank (http://www.ncbi.nlm.nih.gov/), Ribosomal Database Project (RDP-II) (http: //rdp.cme. msu.edu/html/), due to this case we carried out further construction of phylogenetic trees with the nucleotide sequences (http://www.bacterio.net). The analysis included ITS region nucleic acid sequence, the most phylogenetically related organisms.

Mega 3.1 Software was used to construct phylogenetic trees. ClustalW algorithm was used to align the nucleotide sequences; construction of a tree was performed by using the method of the nearest neighbor attachment (Neiighbor-Joining (NJ)) as shown in Fig. 3-5.

Figure 3 shows that the nucleotide sequence of the sample 2 is located on the same branch with the maximum percentage of *Penicillium billaiae*. Considering the maximum percent of analyzed sequence matches in International database of BLAST algorithm and results of phylogenetic analysis indicated that the sample 2 is of *Penicillium billaiae*. Also, the figure shows that the sequence of the sample 4 is located on a branch with a representative of *Penicillium griseofulum* and *Penicillium flexuosum*. The nucleotide sequence of the sample 1 is located on the same branch with the *Penicillium alii-sativi, tardochrysogenum, chrysogenum*. Due to the proximity of these genetic variations the analyzed samples cannot be set using the ITS region. Other additional methods of analysis are required.

Figure 4 shows that the nucleotide sequence of the sample 3 is located on the same branch with the Talaromyces. Due to the proximity of these genetic variations the analyzed samples can not be set using the ITS region. Other additional methods of analysis are required.

Figure 5 shows that the sequence of ITS region 5 is located on one branch with *Aspergillus tubingensis*. Considering the maximum percent of analyzed sequence matches in International database of BLAST algorithm and results of phylogenetic analysis indicated that the sample 5 is *Aspergillus tubingensis*. Also, the figure shows that the sequence of the sample 3 is located on a branch with *Aspergillus aeropaeus*. Considering the maximum percent of analyzed sequence matches in International database of BLAST algorithm and results of phylogenetic analysis indicated that the sample 3 relates to *Aspergillus aeropaeus*.

Thus, genotyping fungal cultures gave an evaluation of genus level. Concerning the genus level there are differences as a culture can belong to certain types

Table 6: The results of fungi genetic identification on the basis of nucleotide sequence analysis of ITS region

No. of culture (1)	Nucleotide sequence (2)	Result of identification (BLAST GeneBank algorithm)		
		No. of gene bank (3)	Name of organism (4)	Coincidence (%, 5)
1	CCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCT CTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTC GGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGG GCCCGCGCCCGCCGAAGACACCCTCGAACTCTGTCTGAAGATTG TAGTCTGAGTGAAAATATAAATTATTTAAAACTTTCAACAACGG ATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT ACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAA CGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGA GCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGT CCTCCGATCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCG CGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAG GCCCGGCCGGCGC	NR_138308.1	*Penicillium tardochrysogenum*	100
		LT558876.1	*Penicillium chrysogenum*	100
		KP120886.2	*Penicillium commune*	100
2	ATTACTGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTC	KU574710.1	*Penicillium bilaiae*	100
	TCTTGTACCATGTTGCTTCGGCGAGCCCGCCTCACGGCCGCCGG	KP096311.1	*Lumnitzera racemosa*	99
	GGGGCATCTGCCCCCGGGCCCGCGCCCGCCGAAGCCCCCTCTGA ACGCTGTCTGAAGATTGCAGTCTGAGCGATAAGCAAAAATTATT TAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAG AACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGT GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCC GGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGG CTTGTGTGTTGGGCCTCCGTCCTCCCCCCGGGGGGACGGGCCCG AAAGGCAGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGC TTTGTCAC	KT692577.1	*Talaromyces flavus*	99
3	GTTCTGTCTTACCCTTGCTTGCTTCGCGCGGGCCCGCCATTCGT	LT220221.1	*Aspergillus europaeus*	99
	GGGCCGCCGGGGGGCATTTCGCCCCCCGGGCCCGCGCCCGCCGGA	KP131641.1	*Aspergillus wentii*	99
	GTACACCAACACGAACTCTGTCTGAAGGTTGCAGTCTGAGTCGA TTTATTTAATCGTTAAAACTTTCAACAACGGATCTCTTGGTTCC GGCATCGATGAAGAACGCATCGAAATGCGATAATTAATGTGAAT TGCAGAATTCATTGAATCATCGAGTCTTTGAACGCACATTGCGC CCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTG CCCTCAAGCACGGCTTGTGTGTTGGGTYGCCGTCCCCGTCCCGG GGGACGGGCCCCAAAGGCAGCGGGGGCACCGCGTCCGGTCCTCG AGCGTATGGGGGTTTGTCACC	HE608161.1	*Aspergillus fumigatus*	99
4	GTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTACC TTGTTGCTTCGGCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTT	KR703615.1	*Penicillium griseofulvum*	99
	ACGCCCCCGGGCCCGCGCCCGCCGAAGACACCCTCGAACTCTGT CTGAAGATTGTAGTCTGAGTGAAAATATAAATTATTTAAAACTT TCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGC GAAATGCGATA CGTAATGTGAATTGCAAATTCAGTGAATCATCG AGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCAT GCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGT TGGGCCCCGTCCTCCGATTCCGGGGGACGGGCCCGAAAGGCAGC GGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACC CGTCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATT	KC411673.1	*Penicillium flexuosum*	99
5	GGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGCATG	KU702706.1	*Aspergillus niger*	99
	TGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGC TGCCTTTCGGGCCCGTCCCCCCGGAGAGGGGGACGGCGACCCAA CACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCA TGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTC GATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTC GCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGA AAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTT CAGACAGTGTTCGTGTTGGGGTCTCCGGCGGGCACGGGCCCGGG GGGCAAAGGCGCCCCCCCGGCGGCCGACAAGCGGCGGGCCCGCC GAAGCACAGGGTATAATAGACACGGATGGGAGGTTGGGCCCAAA GGACCCGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACG	KX649892.1	*Aspergillus tubingensis*	99
6	CAAGGTTTCCGTAGGTGAACCTGGGAAGGATCATTACCGAGTGC GGGCCCTCGCGGCCCAACCTCCCACCCTTGTCTCTATACACCTG	HQ608025.1	*Talaromyces verruculosus*	99
	TTGCTTTGGCGGGCCCACCGGGGCCACCTGGTCGCCGGGGGACG CACGTCCCCGGGCCCGCGCCCGCCGAAGCGCTCTGTGAACCCTG ATGAAGATGGGCTGTCTGAGTACTATGAAAATTGTCAAAACTTT CAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG AAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCG AATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGCAT GCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTGT TGGGTGTGGTCCCCCCGGGGACCTGCCCGAAAGGCAGCGGCGAC GTCCGTCTGGTCCTCGAGCGTATGGGGCTCTGTCACTCGCTCGG GAGGGACCTGCGGGGGTTGGTCACCACCATGTTTT	KT692577.1	*Talaromyces flavus*	99

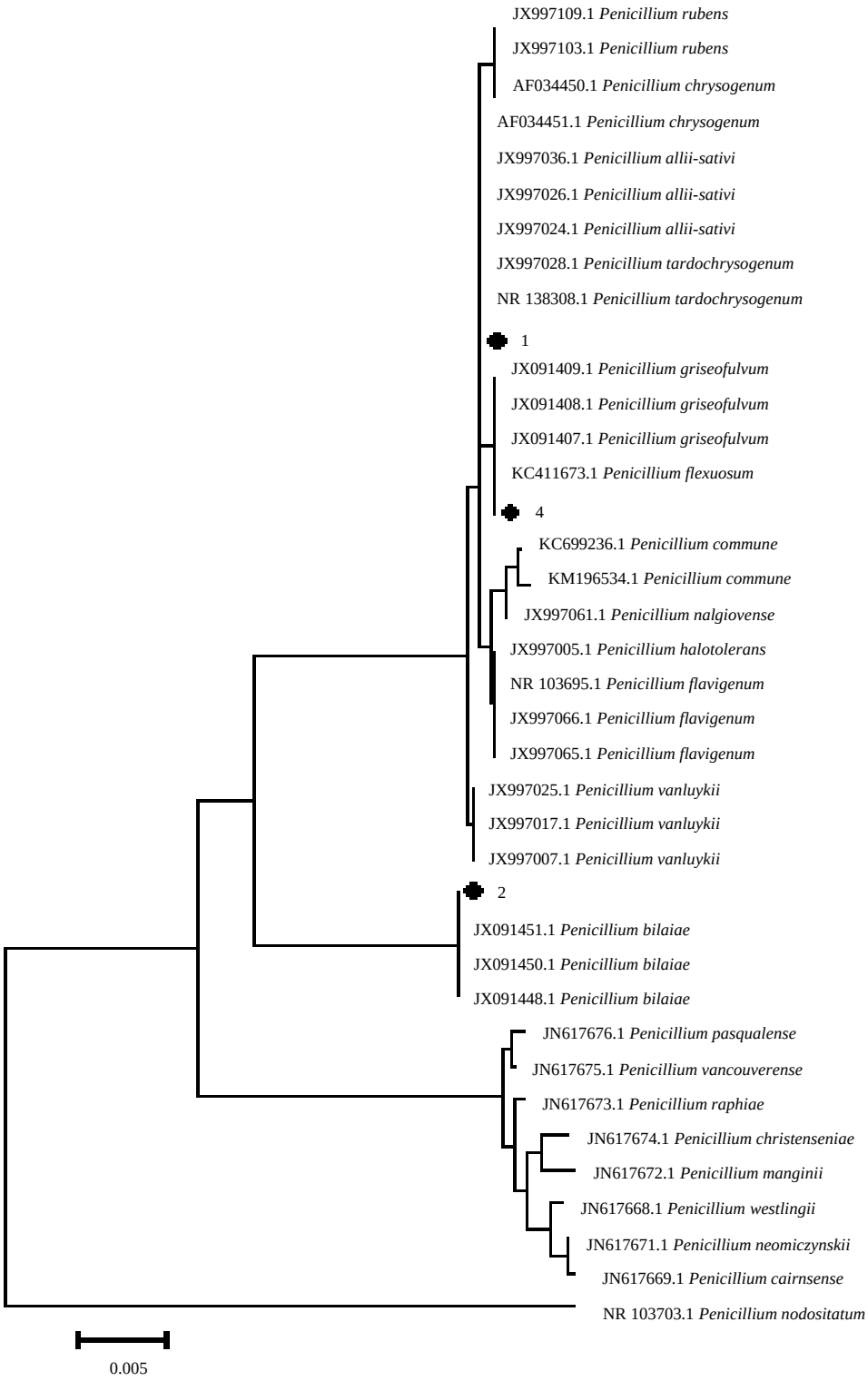

Fig. 3: The phylogenetic tree constructed on ITS region fragment analysis of *Penicillium* genus

simultaneously as shown by the trees (Fig. 3-5) constructed by us. Therefore, for determination of cultures genus level it is necessary to carry out the analysis with phenotypic data.

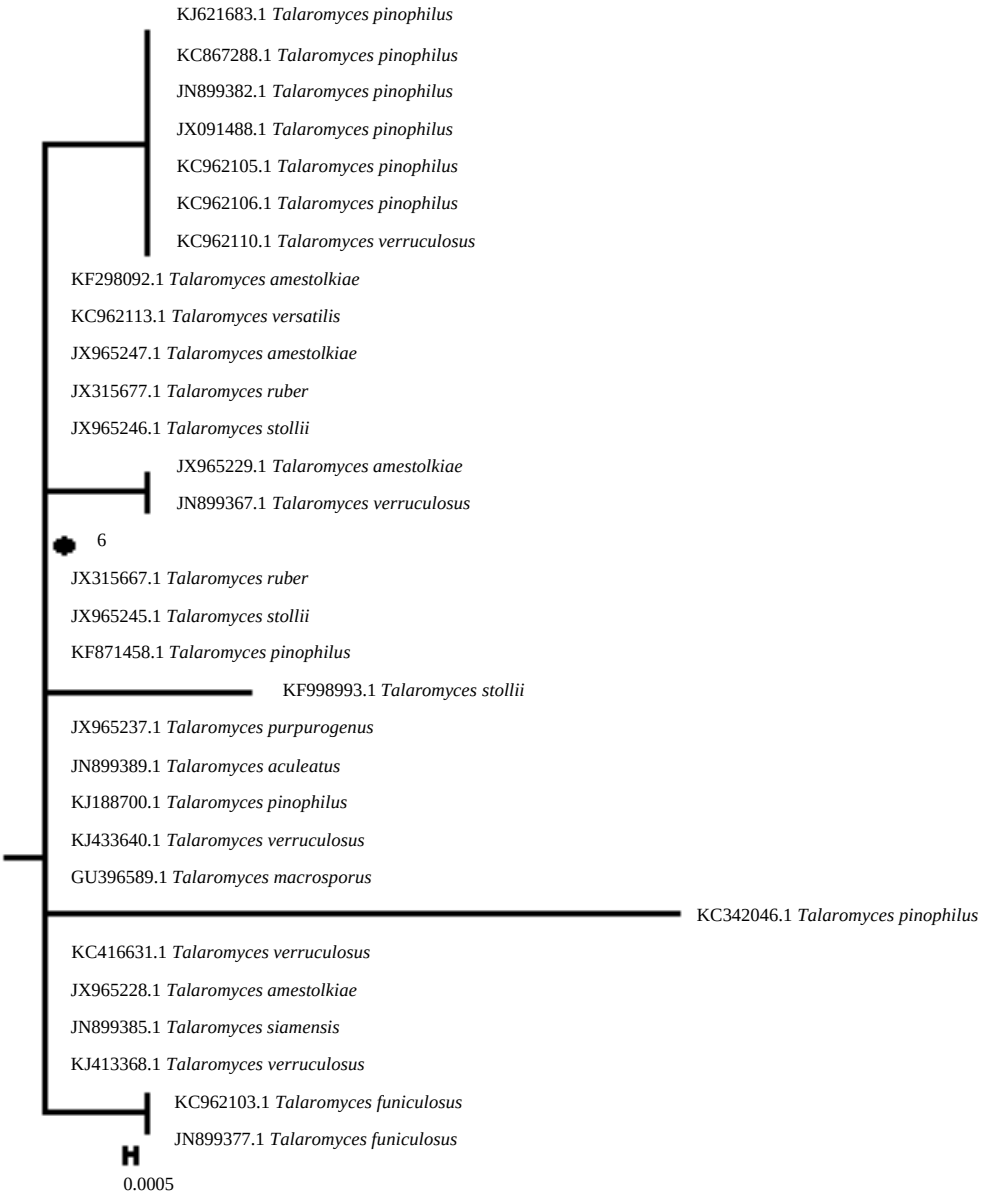

Fig. 4: The phylogenetic tree constructed on ITS region fragment analysis of *Talaromyces* genus

We would like to note that our previous preliminary identification was carried out using the cultural-morphological and physiological-biochemical features (Table 1). Summarized results are presented in Table 7. Thus, by analyzing the results of the identification, it can be noted that in the case of culture 34/307 in both directions of the study the data is distinctive for *Penicillium chrysogenum*. In the case of culture 3 during genotyping it can belong to the three genus: *Penicillium, Lumnitzera, Talaromyces*. According to the culture-morphological, physiological and biochemical features the culture belongs to the *Penicillium genus*. Consequently it is a fungus of the genus *Penicillium* with a specific name-*Bilaiae*. Strain 63/360 may belong to three species of the genus *Aspergillus* but has all features of *Aspergillus fumigatus* that proves the microbiological studies. A similar pattern is typical for strains 282 and 256. In the case of culture 183/1 it is necessary to make additional tests in the future. But we reject that this fungus belongs to the niger genus as the external features are not typical for this type of fungi. Therefore, 6 fungal cultures of microorganisms are

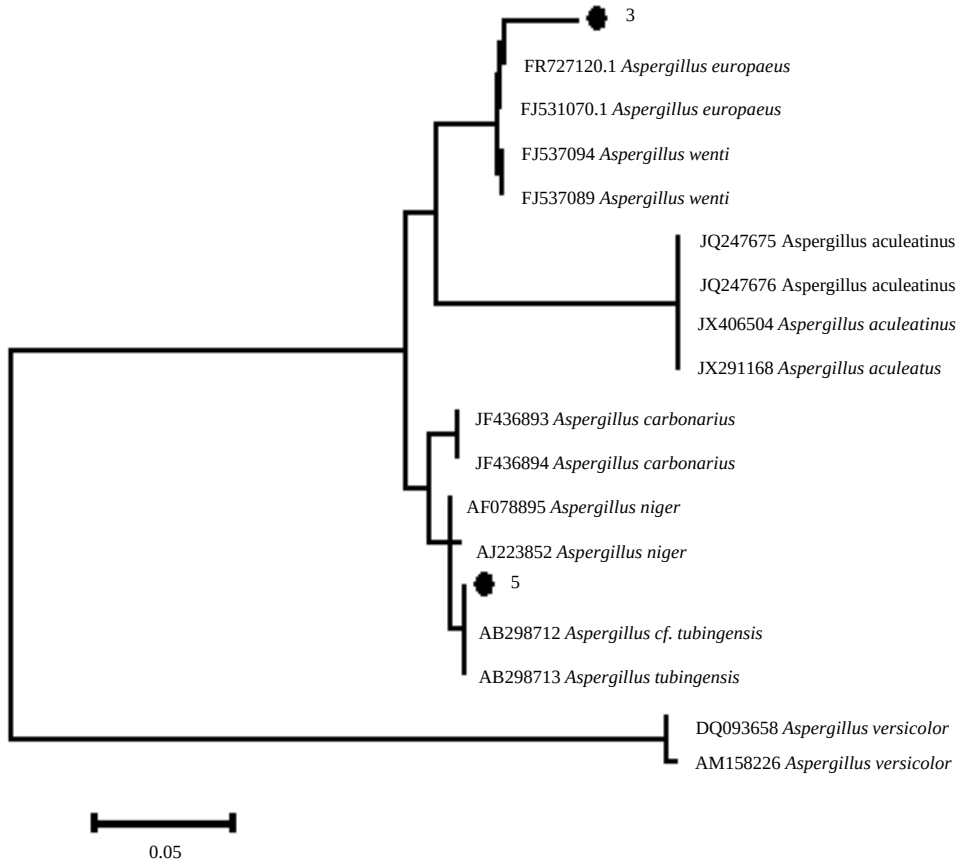

Fig. 5: The phylogenetic tree constructed on ITS region fragment analysis of *Talaromyces* genus

Table 7: Comparison of genotyping and phenotyping results

Designation of cultures	Culture name on phenotype	Culture name on genotype	Results
34/307	*Penicillium chrysogenum*	*Penicillium tardochry-sogenum/Penicillium chrysogenum/Penicillium commune*	*Penicillium chrysogenum*
3	*Penicillium* sp.	*Penicillium bilaiae/Lumnitzera racemosa/Talaro-myces flavus*	*Penicillium bilaiae*
63/360	*Aspergillus fumigatus*	*Aspergillus europaeus/ Aspergillus wentii/Aspergillus fumigatus*	*Aspergillus fumigatus*
282	*Penicillium griseofulvum*	*Penicillium griseofulvum/ Penicillium flexuosum*	*Penicillium griseofulvum*
183/1	*Aspergillus awamory*	*Aspergillus niger/Aspergillus tubingensis*	*Aspergillus niger/Aspergillus tubingensis*
256	*Talaromyces flavus*	*Talaromyces verruculosus/Talaromyces flavus*	*Talaromyces flavus*

genotyped. The results of the genetic study can be used as molecular and biological characterization of strains.

CONCLUSION

In this study we isolated active fungal culture from grain. These 27 samples will be added to the collection of fungi in Institute. Proteolytic activity of fungi was studied, in particular the ability to liquefy gelatin, casein. We have screened and selected the most active strains: *P. chrysogenum* 34/307, *Penicillium* sp. 3, *P. griseofulvum* 282, *A. fumigatus* 63/360, *A. awamory* 183/1, *T. flavus* 256 for genotyping. Phenotypic and genotypic features set their genus and species identity. For further studies the most active strains of fungi was selected such as *Penicillium bilaiae* 3, *Penicillium chrysogenum* 34/307, *Aspergillus fumigatus* 63/360.

REFERENCES

Anitha, T.S. and P. Palanivelu, 2013. Purification and characterization of an extracellular keratinolytic protease from a new isolate of Aspergillus parasiticus. Protein Expression Purif. 88: 214-220.

Clarridge, J.E. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease. Clin. Microbiol. Rev. 17: 840-862.

Clayton, R.A. G. Sutton, J.P.S. Hinkle, C. Bult and C. Fields, 1995. Intraspecific variation in small-subunit rRNA sequences in GenBank: Why single sequences may not adequately represent prokaryotic taxa. Intl. J. Syst. Evol. Microbiol. 45: 595-599.

De Souza, P.M. M.L. de Assis Bittencourt, C.C. Caprara, M. de Freitas and R.P. Coppini de Almeida *et al*., 2015. A biotechnology perspective of fungal proteases. Brazil. J. Microbiol. 46: 337-346.

Dias, D.R. D.M. Vilela, M.P.C. Silvestre and R.F. Schwan, 2008. Alkaline protease from Bacillus sp. isolated from coffee bean grown on cheese whey. World J. Microbiol. Biotechnol. 24: 2027-2034.

Do, T.T. D.T. Quyen and T.N. Nguyen, 2013. Molecular characterization of a glycosyl hydrolase family 10 xylanase from Aspergillus niger. Protein Expression Purif. 92: 196-202.

Germano, S. A. Pandey, C.A. Osaku, S.N. Rocha and C.R. Soccol, 2003. Characterization and stability of proteases from Penicillium sp. produced by solid-state fermentation. Enzyme Microb. Technol. 32: 246-251.

Huch, M. A. Hanak, I. Specht, C.M. Dortu and P. Thonart *et al*., 2008. Use of lactobacillus strains to start cassava fermentations for gari production. Intl. J. Food Microbiol. 128: 258-267.

Kuddus, M. and P.W. Ramteke, 2012. Recent developments in production and biotechnological applications of cold-active microbial proteases. Crit. Rev. Microbiol. 38: 330-338.

Kumar, C.G. and H. Takagi, 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol. Adv. 17: 561-594.

Kumar, S. K. Tamura and M. Nei, 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5: 150-163.

Kumari, M. A. Sharma and M.V. Jagannadham, 2012. Religiosin B, a milk-clotting serine protease from Ficus religiosa. Food Chem. 131: 1295-1303.

Li, C. Z. Yang, R.H.C. Zhang, D. Zhang and S. Chen*et al*., 2013. Effect of pH on cellulase production and morphology of Trichoderma reesei and the application in cellulosic material hydrolysis. J. Biotechnol. 168: 470-477.

Netrusov, A.I. M.A. Egorova and L.M. Zakharchuk, 2005. Netrusov Practical Work on Microbiology. New York University, New York, USA. Pages: 608.

Ogbonnaya, N. and A. Odiase, 2012. Influence of media composition on the production of alkaline a-amylase from Bacillus subtilis CB-18. Acta Scientiarum Polonorum Technol. Aliment. 11: 231-238.

Pandey, A. P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh and R. Mohan, 2000. Advances in microbial amylases. Biotechnol. Applied Biochem. 31: 135-152.

Pascoal, A.M. S. Mitidieri and K.F. Fernandes, 2011. Immobilisation of a-amylase from Aspergillus niger onto polyaniline. Food Bioprod. Process. 89: 300-306.

Patil, N.S. S.R. Waghmare and J.P. Jadhav, 2013. Purification and characterization of an extracellular antifungal chitinase from Penicillium ochrochloron MTCC 517 and its application in protoplast formation. Process Biochem. 48: 176-183.

Rao, M.B. A.M. Tanksale, M.S. Ghatge and V.V. Deshpande, 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62: 597-635.

Rodarte, M.P. D.R. Dias, D.M. Vilela and R.F. Schwan, 2011. Proteolytic activities of bacteria, yeasts and filamentous fungi isolated from coffee fruit (Coffea Arabica L.). Acta Scientiarum. Agron. 33: 457-464.

Samson, R.A. and J.I. Pitt, 2000. Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification. Harwood Publishers, Amsterdam, Netherlands, ISBN:90-5823-159-3, Pages: 510.

Sanni, A.I. J. Morlon-Guyot and J.P. Guyot, 2002. New efficient amylase-producing strains of Lactobacillus plantarum and L. fermentum isolated from different Nigerian traditional fermented foods. Int. J. Food Microbiol. 72: 53-62.

Tavano, O.L. 2013. Protein hydrolysis using proteases: An important tool for food biotechnology. J. Mol. Catal. B. Enzymatic, 90: 1-11.

Tomar, R. R. Kumar and M.V. Jagannadham, 2008. A stable serine protease, wrightin, from the latex of the plant Wrightia tinctoria (Roxb.) r. br.: Purification and biochemical properties. J. Agric. Food Chem. 56: 1479-1487.

Werle, E. C. Schneider, M. Renner, M. Volker and W. Fiehn, 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res. 22: 4354-4355.

Zhang, Q. R. Kennon, M.A. Koza, K. Hulten and J.E. Clarridge, 2002. Pseudoepidemic due to a unique strain of Mycobacterium szulgai: Genotypic, phenotypic and epidemiological analysis. J. Clin. Microbiol. 40: 1134-1139.