UV- and EMS- induced mutations affecting synthesis of alkaloids and lipase in *Penicillium roquefortii*

(Received: 13.03.2007; Accepted: 28.03.2007)

Ahmed M. EL-Bondkly* and Abeer A. Keera**
*Applied Microbial Genetics Laboratory, Genetics & Cytology Department, **Microbial Chemistry Department, National Research Center, Giza, Egypt.
E-mail: ahmed_bondkly @yahoo.com

ABSTRACT

Mutants of a Penicillium roquefortii strain were obtained by ultraviolet irradiation (UV) and ethylmethansulfonate (EMS). Based on alkaloid production, the mutant strains could be divided into four groups: (1) unable to synthesize alkaloids, (2) with a non functional chain of clavin formation, (3) with roquefortine and 3,12-dihydroroquefortine that were not found in the alkaloid fraction and (4) with new compounds, that were not shown in the wild type strain. These data indicate the presence of not less than three different pathways of alkaloids formation in Penicillium roquefortii. On the other hand, all selected mutants produced more lipase than the wild type strain, when using olive oil as a substrate; when cotton seed oil was used many mutant strains produced less lipase activity than the parent strain.

Key words: *Penicillium roquefortii, alkaloids, lipase, mutants.*

INTRODUCTION

Penicillium roquefortii is the universally studied representative of the genus *Penicillium*. First, this fungus is used in the food industry for manufacturing the so-called blue cheeses Roquefort, Danablu and Gorgonzola. Second, it is among the most frequent contaminants of various foodstuffs, including rice, meat, vine, grain, silage, dairy products, etc…,. Therefore, the ability of this fungal species to produce mycotoxins has attracted much attention (Vinokurova *et al.,* 2001 and 2003).

Penicillium roquefortii synthesizes mycotoxins belonging to diketopiperazine (roquefortine and 3, 12- dihydroroquefortine) and clavine alkaloids (chanoclavine, isofumigaclavines A and B and festuclavine) (Boichenko *et al.,* 2002; Bringmann *et al.*, 2005; Zelenkova *et al.,* 2003). Roquefortine which is a strong neurotoxin and antibiotic recently attracted much attention. The formation of roquefortine in food products and feed is of epidemiological importance. Moreover, this metabolite is of great importance in veterinary medicine for its possibility to cause toxicosis. Roquefortine is used as a chemotaxonomic marker of *Penicillium* fungi (Buzilova *et al.*, 2000).

Lipases (glycerol ester hydrolases EC 3.1.1.3) are produced by a wide-spread number of microorganisms including bacteria, fungi and yeasts (Dai and Xia, 2006; Dalmau *et al.,* 2000). In recent years, research on microbial lipases has increased because of their practical applications in industry, as hydrolysis of fats, production of fatty acids and food additives, synthesis of esters and peptides, resolution of racemic mixtures, or additives in detergents (Makhsumkhanov *et al.,* 2003).

Microbial production of alkaloids depends on the activity and stability of the producers, which can be enhanced by induced mutagenesis. The effect of mutations on the biosynthesis of alkaloids with various structures is of applicable and theoretical importance. Because genetics of alkaloids formation has not been widely studied, this work designs the effect of mutations using physical (UV-light) and chemical (EMS) mutagens on the biosynthesis of alkaloids and lipase to be produced by strains of *Penicillium roquefortii*.

MATERIALS AND METHODS

A culture of *Penicillium roquefortii* was obtained from National Research Center (Egypt) and maintained on potato dextrose agar (PDA) slants. For UV-mutagenesis, spores obtained from 5-day old cultures were resuspended in sterile distilled water and exposed to UV-light (254 nm) from Philips T-UV-30 W lamp source at a distance of 25 cm for 5, 10 and 15 min. The treated conidia were put in dark for one hr and transferred to dishes with glucose-potato agar. On the other hand, conidiospores of 5-day old cultures were subjected to ethylmethansulfonate (EMS) mutagenesis. Spores were collected and incubated in phosphate buffer pH 7.0 containing 100 µl / ml EMS (Sigma Co.,) for 30 and 60 min. Spores were then washed with sterile distilled water and serial dilutions were prepared for inoculating glucose potato agar.

The wild type strain and mutants were grown in Abe′s medium described by Buzilova *et al.* (2000), which is optimal for alkaloids formation. The medium contained (g/l): mannitol, 50.0; succinic acid, 5.4; $MgSO_4$. H_2O, 0.3 and KH_2PO_4, 1.0, pH was adjusted to 5.2 with 25 % NH_4OH. The medium was inoculated with 10 % spore suspensions. The fungal spores were grown in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium. The inoculated flasks were kept on a rotary shaker (220 rpm) at 25°C for eight days. Thereafter, metabolites were isolated from filtrate and mycelium of the cultures by chloroform extraction. The chloroform extracts were dried over anhydrous Na_2 SO_4, filtered and evaporated to dryness.

Separation of alkaloid fractions into components was performed by thin –layer chromatography (TLC) on silica UV-254 plates (Fluka Chemie Co.) in a mixture of chloroform – methanol – 25 % NH_4OH (90: 10: 1). The components were visualized by optical absorption in the UV region and staining with Ehrlich′s reagent. Alkaloids content was estimated semiquantitatively by visual comparison of size and intensity of colored chromatographic spots. Afterwards, alkaloids content in chloroform extracts were measured and analyzed by high – performance liquid chromatography (HPLC, Shimadzu) in a liquid chromatograph (column, Lura CN) equipped with refractive index detector, elution rate, 1.0 ml/min and solvent system, methanol – water – 25 % NH_4OH (60 : 40 : 0.036 vol. %) as described by Vinokurova *et al.* (2001).

Lipase production by *Penicillium roquefortii* was monitoved in liquid Raistrick′s medium containing 1.0 % soybean flour, 0.6 % $NaNO_3$, 0.1 % KCl, 0.85 % K_2HPO_4, 0.025 % $MgSO_4$.$7H_2O$, 0.1 % $CaCO_3$, 1.0 % cotton seed or olive oil and tap water. The fermentation conditions were similar to those mentioned for alkaloids formation. The activity of extracellular lipase was determined as described by Kilcawley *et al.* (2002). Activity was expressed in units where one unit releases one µmol of ρ-nitrophenol.

RESULTS AND DISCUSSION

Induction of genetic variabilities

Physical (UV-light) and chemical (EMS) mutagens proved to be powerful in inducing a wide range of genetic variabilities, although the mutants isolated after their use were, in some cases, unstable.

On the basis of these findings, the two mutagens, UV-irradiation and EMS were applied in different doses for inducing mutations in the mould *P. roquefortii*. Results (Fig. 1) show the expected gradual increase of the lethality which was associated with the increase of the mutagen dosage. Meanwhile, after the application of UV-light, the highest numbers of the morphological variants obtained were induced as a result of 10 min exposure (15.3 %), followed by those obtained from the suspensions exposed to 5 and 15 min (10.5 and 5.2 %), respectively. On the other hand, the survival percentages sharply decreased by increasing EMS doses. It was 20.8 % due to 100 µl / ml treatment for 30 min, and then sharply dropped down with the increasing time of treatment. When the dose 100 µl / ml of EMS was applied for 60 min, the survival percentage was 7.2. In addition, the number of morphological variants were 6.5 % and 12.5 % following 100 µl / ml for 30 min and 100 µl / ml for 60 min EMS treatments, respectively.

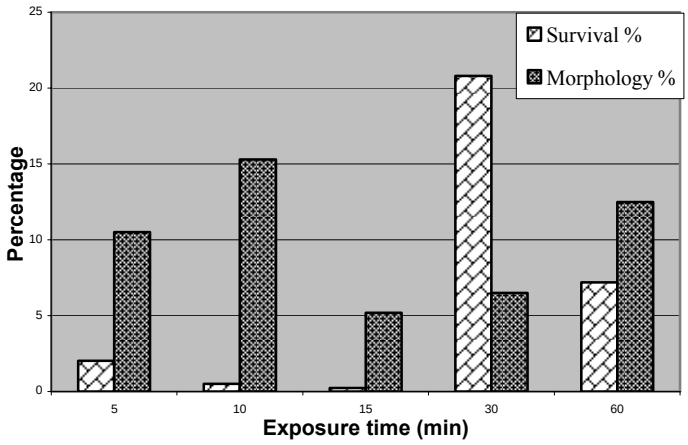

Fig. (1): Percentages of both survival and morphological variants for each dose of the two mutagens (5, 10 and 15 min for UV treatment as well as 30 and 60 min for 100 µg / ml EMS treatment).

Formation of alkaloids by mutant strains of *P. roquefortii*

The *P. roquefortii* culture synthesizes some secondary compounds of the diketopiperazine (roquefortine and 3, 12 – dihydroroquefortine) and clavinet groups (chanoclavine, isofumigaclavine A and B, agroclavine and festuclavine) (Buzilova *et al.*, 2000).

In this study, a number of 94 mutant strains displaying differences from the wild type strain after mutagenesis induced by UV-light and EMS agent were selected. The color and shape of developed colonies and growth in liquid and solid media exhibited considerable variations. Conidiogenesis was completely suppressed after high doses of mutagenic treatments. Moreover, the majority of mutants displayed significant changes in the contents of some components of the alkaloid mixture. Morphological mutants were accompanied by significant changes in alkaloid formation. However, strains in which alkaloid formation was particularly the same as in the wild type were found. On the other hand, the absence of correlation between the level of roquefortine and yellow color of mycelium of *P. roquefortii* was shown.

According to alkaloids synthesis, the mutants obtained could be divided into four groups. The mutant strains that almost completely lost their ability to synthesis alkaloids belong to the first group (Figs. 2 and 3). However, such strains have rarely appeared, probably because of the relative independence of the initial stages of alkaloids biosynthesis of different structural groups in *P. roquefortii*. Besides, the mutant strains (5/18, 5/20 and 60/20) that inherited the desired activity had no considerable specific abnormalities of development. The second group included mutants with a non-functional chain of clavin formation. This group was represented by two forms. The mutant (10/2) had no clavin – forming activity. However, they did not display any significant increase in the level of roquefortine. Clavine alkaloids specific to the wild type were not found in mutants 60/16 and 30/1. Alkaloids such as cyclopenine, cyclopenol and viridicathine which are unusual in the initial strain were formed. These results suggest that mutation of the gene controlling the initial stage of synthesis of clavinet alkaloids leads to an increase in the level of tryptophane, the common precursor of alkaloids of all structural groups in *P. roquefortii*. This increase activated cyclopeninviridicathine biosynthesis which was usually blocked in the wild-type strain. The third group of mutants was characterized by the opposite tendency. Roquefortine and 3, 12 – dihydroroquefortine were not found in the alkaloid fraction of the mutants (30/9 and 30/18). In addition, alkaloids synthesis by the mutants (5/12, 5/13, 15/3 and 15/5) belonged to the clavine type. Mutation of the genes controlling iso-funigaclavines may be suggested. The fourth group of mutants produced new compounds, not shown in the wild type strain. The mutant strains 5/12 and 5/13 formed new compounds as shown in Figs. (2 and 3).

Thus, these data indicated the presence of not less than three different pathways of alkaloids formation in *P. roquefortii*. There is a pathway of viridicathine synthesis in addition to two pathways described above (Fig. 4). It is difficult to conclude whether these changes result from mutation of the gene involved directly in alkaloid formation or are induced by rearrangements of other parts of the genome. In addition, these data raise the problem of the relationship between the functioning pathways of alkaloid biosynthesis and potential, genetically determined pathways in *P. roquefortii*.

The data obtained were match with those of Buzilova *et al.* (2000) who isolated 400 mutant strains displaying differences from the wild-type strain after mutation induction in *P. roquefortii* VKM F-141 and *P. fellutanum* VKM F-1073 by UV light and chemical agents. The mutants could be divided into three groups; (1) unable to synthesize alkaloids, (2) with a high rate of biosynthesis, (3) with changed alkaloid composition. Moreover, Vinokurova *et al.* (2001) isolated new isomers of clavinet alkaloids from the collection of mutant strains from *P. roquefortii* Thom1906. It was demonstrated that the collected strain produces isomers of agroclavine and epoxyagroclavine, whereas the mutant strain synthesizes isomers of fumigaclavines A and B, festuclavine and chanoclavine.

In spite of the limited interpretation, these data provide the basis for further studies of the molecular mechanisms of alkaloids formation and the role of mycotoxins in the physiology of producers. Moreover, the creation of new active producers of specific mycotoxins is of great importance to biochemical and pharmacological studies and food industry, as well as sanitary and epidemic monitoring.

Fig. (2): Thin-layer chromatography of chloroform extracts from culture liquids of mutants and fungal parent strain after Ehrlich's reagent staining.

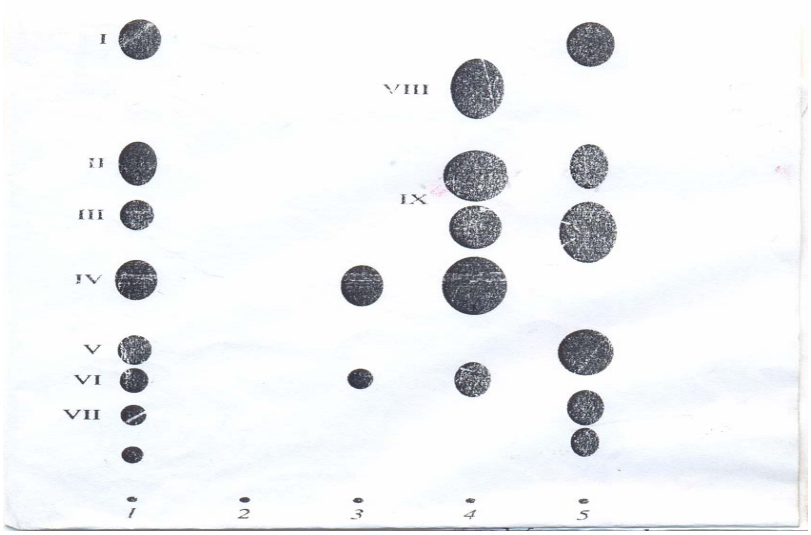

Fig. (3): Biogram representing chloroform extracts from culture liquids of mutants. (1) P. roquefortii; (2) 5/18, 5/20 and 60/20; (3) 10/2; (4) 60/16 and 30/1; (5) 30/9 and 30/18. I, isofumigaclavine A; II, agroclavine; III, festuclavine; IV, roquefortine; V, isofumigaclavine; VI,3,I2- dihydroroquefortine; VII, chanoclavine; VIII, viridicathine; IX, cyclopenine and cyclopenol.

Lipase productivity by mutant strains of *P. roquefortii*

Twenty five isolates selected on the basis of difference in alkaloids formation and tested for lipase productivity on different oil sources (olive and cotton seed). Table (1) presents the selected mutants and lipase productivity compared with the *P. roquefortii* parent strain. Out of 25 isolates, 17 were isolated after UV-light treatments (8, 4 and 5 isolates after 5, 10 and 15 min exposure time, respectively) and 8 mutants induced after EMS treatments (4 and 4 mutants after 100 µl / ml for 30 min and 60 min, respectively). All isolates produced more lipase than the parent strain on olive oil, while when cotton seed oil was used many isolates produced less lipase activities than the parent strain. Furthermore, one mutant (5/8) did not

show any lipase activity on cotton seed oil. On the other hand, two mutants 5/12 and 5/13 gave high lipase activities, they showed a yield of 2.5 and 0.5 U/ml on olive oil and 2.0 and 3.0 U/ml on cotton seed oil, respectively. Whereas, mutant 5/9 proved to give 1.0 U/ml on olive and cotton seed oil. Different investigators studied the conditions affect lipase activity, but there are little studies on genetic improvement. As previously mentioned *Penicillium roquefortii* is used in food industry for manufacturing the so-called blue cheeses Roquefort, Danablu and Gorgonzola so that it is important to study and improve its lipase activity (Tianwei *et al.*, 2004). Makhsumkhanov *et al.* (2003) used Raistrick's medium of their own modification and found that the lipase activity increased three to four times.

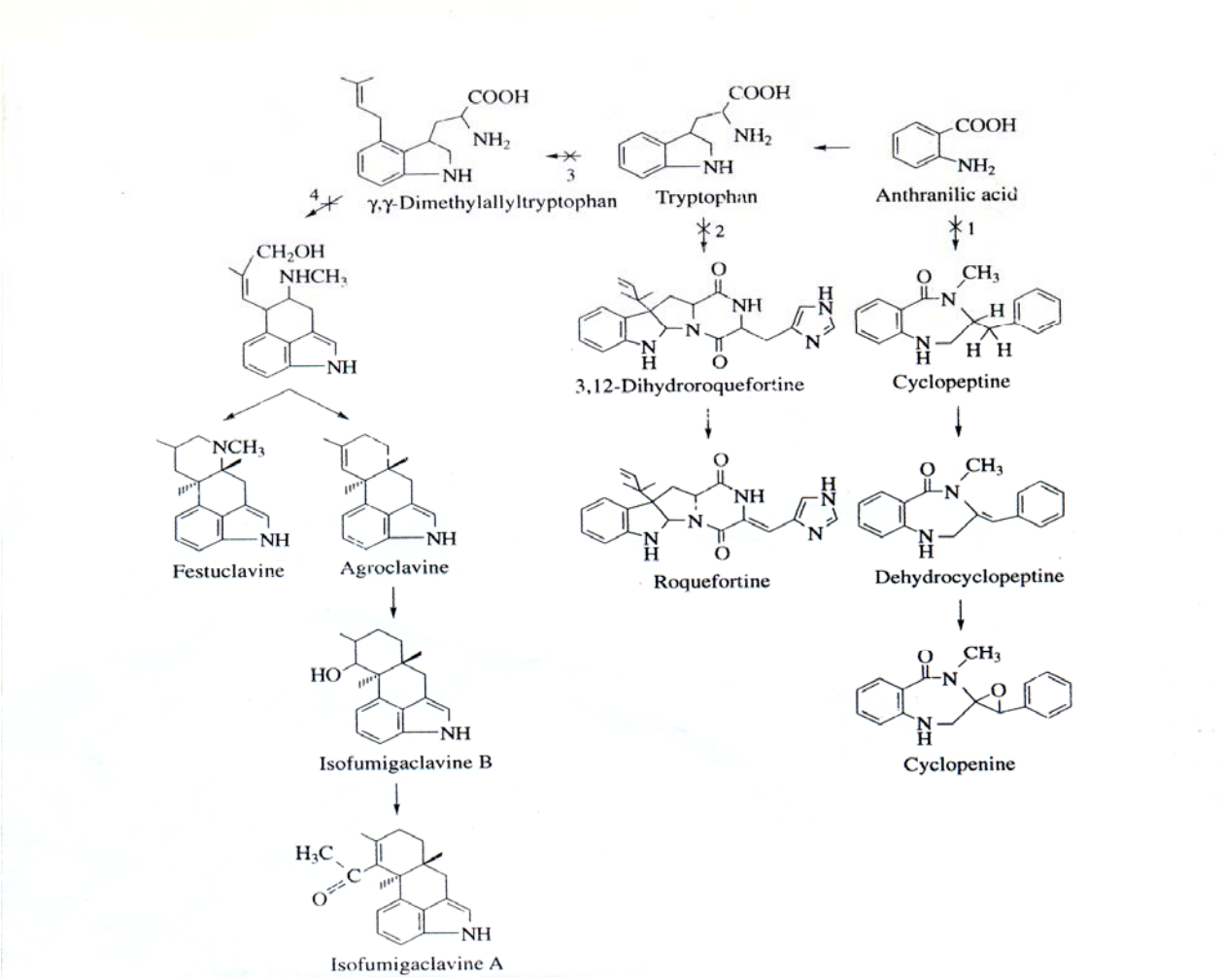

Fig. (4): Scheme for alkaloids biosynthesis in P. roquefortii. (1) block of synthesis of quinoline and benzodiazepine alkaloids, (2) block of synthesis of diketopiperazine alkaloids, (3, 4) possible localization of the block of synthesis of clavine alkaloids (Buzilova et al., 2000).

Table (1): Lipase activity by mutant strains of P. roquefortii using olive and Cotton seed oils compared with the parent strain.

Isolate	Lipase activity			
	Olive oil		Cottonseed oil	
	U/ml	% From parent	U/ml	% From parent
Parent	0.1	100.0	0.5	100.0
5/7	0.5	500.0	0.7	140.0
5/8	0.2	200.0	0.0	0.0
5/9	1.0	1000.0	1.0	200.0
5/12	2.5	2500.0	2.8	560.0
5/13	0.5	500.0	3.0	600.0
5/18	0.2	200.0	0.2	40.0
5/20	0.3	300.0	0.2	40.0
5/23	0.4	400.0	0.5	100.0
10/2	0.3	300.0	0.3	60.0
10/9	0.4	400.0	0.3	60.0
10/12	0.4	400.0	0.5	100.0
10/16	0.3	300.0	0.3	60.0
15/2	0.4	400.0	0.5	100.0
15/3	0.2	200.0	0.2	40.0
15/5	0.3	300.0	0.2	40.0
15/8	0.4	400.0	0.3	60.0
15/11	0.2	200.0	0.2	40.0
30/1	0.3	300.0	0.3	60.0
30/9	0.2	200.0	0.3	60.0
30/18	0.4	400.0	0.5	100.0
30/20	0.4	400.0	0.3	60.0
60/13	0.2	200.0	0.2	40.0
60/16	0.3	300.0	0.3	60.0
60/20	0.6	600.0	0.6	120.0
60/21	0.3	300.0	0.2	40.0

REFERENCES

Boichenko, D. M.; Zelenkova, N. F.; Arinbasarov, M. U. and Reshetilova, T. A. (2002). Optimization of the medium and cultivation conditions of *Penicillium roquefortii* f39 producing the diketopiperazine alkaloid roquefortine. Appl. Biochem. Microbiol., 38 (3): 222 – 225.

Bringmann, G.; Gerhard, L.; Tobias, A. M. G.; Hideyuki, T.; Jör, M.; Katja, M.; Stefan, S.; Karsten, S.; Rüdiger, S.; Jutta, W.; Johannes, F. I.; Sanja, P.; Olexandra, B. and Werner, E.G. M. (2005). The first sorbicillinoid alkaloids, the antileukemic sorbicillactones A and B, from a sponge – derived *Penicillium chrysogenum* strain. Tetrahedron, 61: 7252 – 7265.

Buzilova, I. G.; Boichenko, D. M.; Boichenko, L. V.; Zelenkova, N. F.; Arinbasarov, M. U.; Baskunov, B. P. and Reshetilova, T. A. (2000). Effect of mutation on synthesis of alkaloid by *Penicillium roquefortii* VKM F-141 and *P. fellutanum* VKM F-1073. Appl. Biochem. Microbiol., 36 (3): 276 – 281.

Dai, Da-Zhang and Xia Li-Ming (2006). Resolution of (R, S)-2-octanol by *Penicillium expansum* PED-03 lipase immobilized on modified ultrastable-Y molecular sieve in microaqueous media. Process Biochemistry, 41: 1455 – 1460.

Dalmau, E.; Montesinos, J. L.; Lotti, M. and Casas, C. (2000). Effect of different carbon sources on lipase production by *Candida rugosa*. Enzyme and Microbial Technology, 26: 657 – 663.

Kilcawley, K. N.; Wilkinson, M. G. and Fox, P. F. (2002). Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. Enzyme and Microbial Technology, 31: 310 – 320.

Makhsumkhanov, A. A.; Yakubov, I. T. and Davranov, K. (2003). Conditions for cultivation of the fungus *Penicillium melinii* UzLM-4 and its biosynthesis of lipases. Appl. Biochem. Microbiol., 39 (1): 40 – 43.

Tianwei, Tan; Zhang, Mu; Jiali, Xu and Zhang, Jun (2004). Optimization of culture conditions and properties of lipase from *Penicillium camembertii* Thom PG-3. Process Biochemistry, 39: 1495 – 1502.

Vinokurova, N. G.; Boichenko, D. M.; Baskunov, B. P.; Zelenkova, N. F.; Vepritskaya, I. G.; Arinbasarov, M. U. and Reshetilova, T. A. (2001). Minor alkaloids of the fungus *Penicillium roquefortii* Thom 1906. Appl. Biochem. Microbiol., 37 (2): 184 – 187.

Vinokurova, N. G.; Boichenko, L. V. and Arinbasarov, M. U. (2003). Production of alkaloids by fungi of the genus *Penicillium* grown on wheat grain. Appl. Biochem. Microbiol., 39 (4): 403 – 406.

Zelenkova, N. F.; Vinokurova, N. G. and Arinbasarov, M. U. (2003). Analysis of secondary metabolites of microscopic fungi of the genus *Penicillium* by chromatographic techniques. Appl. Biochem. Microbiol., 39 (1): 44 – 54.

الملخص العربي

تأثيرالطوافر المستحدثة بإستخدام ال UV و EMS على تخليق فطر البنسيليوم ريكفورتاى للألكالويدات و الليبيز

أحمد محمد البندقلى * و عبير على كيره **

* قسم الوراثة و السيتولوجى – ** قسم كيمياء الكائنات الدقيقة – المركز القومى للبحوث – الدقى – الجيزة – مصر .

تم استحداث مجموعة من الطوافر فى فطر البنسيليوم ريكفورتاى بإستخدام الأشعة ما فوق البنفسجية كمطفر طبيعى و الإثيل ميثان سلفونيت كمطفر كيميائى. ووجد أن إنتاجية الطوافر المستحدثة من الفطر قسمت إلى أربع مجموعات المجموعة الأولى و هى غير قادرة على إنتاج الألكالويدات كما فى العزلات (5/18 و5/20 و 60/20) بينما فى المجموعة الثانية فجميع العزلات التى شملتها لم تستطع تكوين سلسلة الكلافين الفعالة مثل (10/2) . و بالنسبة للمجموعة الثالثة فالعزلات التى تنتمى اليها لم تستطع تكوين الروكفورتين و احد مشتقاته وهو 3 , 12 – داي هيدروروكفورتين مثل العزلتين (30/9 و30/18) اما المجموعة الأخيرة فوجد ان لها القدرة على تخليق بعض المركبات الجديدة و التى لم تظهر فى السلالة البرية كما فى العزلتين (5/12 و 5/13) . اشارت النتائج ايضا إلى وجود ثلاثة خطوات مختلفة على الأقل لتكوين الألكالويدات فى فطر البنسيليوم ريكفورتاى. و عند دراسة انتاجية بعض هذه العزلات من انزيم الليبيز بإستخدام أثنين من الزيوت المختلفة و هما زيت الزيتون و زيت بذرة القطن وجد أن جميع العزلات التى تم اختيارها أعطت نشاطاً لإنزيم الليبيز أعلى من السلالة البرية عند نموها على زيت الزيتون بينما العديد من هذه العزلات اعطت نشاط اقل من السلالة البرية بالنسبة لإنزيم الليبيز عندما نميت على زيت بذرة القطن.