INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
06-178/MME/2008/10–1–42–46
http://www.fspublishers.org

Full Length Article

Genetic Improvement of Dextranase Production by *Penicillium funiculosum* via Mutation Induction and Protoplast Fusion

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ABSTRACT

The fungal strain *Penicillium funiculosum* NRRL- 6014 was exposed to UV-irradiation at 20 cm distance from the lamp for 4, 6, 8, 10 and 15 min. About 100 mutants were isolated as auxotrophic or/and morphological variants and screened for dextranase activity qualitatively. Forty mutants proved to be enzyme producers and were chosen for quantitative determination of the enzyme. Results indicated that 30 mutants produced the enzyme more than the wild type, six of which gave dextranase activity two fold or more than the original parent, whereas the remaining 10 mutants produced the enzyme less than the wild type strain. Four different mutants were chosen for protoplast fusion, according to enzyme activity, antifungal agents response and morphological variants, in three cross combinations (a), (b) and (c) in which mutant No. 18 was common parent. A total of 19, 7 and 10 stable fusants were selected from cross combinations (a), (b) and (c), respectively. Dextranase activity of all fusants exceeded that of the wild type strain by about 50 - 250%. The results indicated that fusants from cross combination involving high enzyme productive strains showed enzyme activity more than those of any other crosses.

Key Words: *Penicillium funiculosum*; Mutations; Protoplast fusion; Dextranase; Enzymesg

INTRODUCTION

The presence of dextran in sugar cane mills is a source of serious problems due to the high viscosity they impose on sugar cane juice, as well as deposits of insoluble polymers on the processing equipment. Dextran is a glucose polymer synthesized from sucrose by the enzyme dextransucrase. One of the solutions to contamination problem is the use of dextranase, which hydrolyses the dextran rapidly, reduces the molecular mass and hence the viscosity of the juice. Dextranase has been used, as a toothpaste additive for dental care, since it is well known that dextran is involved in dental plaque formation. Fungi are the most widely distributed microorganisms with dextranolytic activity, especially the species of the genus *Penicillium*. Production of dextranase by *P. funiculosum* was recorded by Szczodrak *et al*. (1994).

Many reports about the induction of higher enzyme producer mutation were published. For example, Hoffman and Wood (1985) induced a mutant strain of *Penicillium funiculosum* by UV irradiation, which was able to saccharify the straw more than its wild type strain indicating that this mutant over-producing cellulose enzyme. Szczodrak *et al*. (1994) irradiated a strain of *P. notatum* by UV-light and selected some dextranase-overproducing mutants, which gave enzyme activity higher than their parental strain by 45 to 131%. The most active mutant strain showing dextranase activity over twice (231%) as high as the wild type strain was stable in subsequent subcultures. Kim and Day (1994) isolated a dextranase constitutive mutant of *Lipomyces starkeyi,* which produced more enzyme (when grown on 1% dextran) than the parental strain. Furthermore, Halos *et al*. (1989) used intergeneric protoplast fusion between *P. funiculosum* and *Trichoderma reesei* and obtained fusants, which showed cellulolytic activity lower than those of their parental strains. However, preliminary results with the second generation (progeny of the fusants) indicated higher CMCase and Fpase activities than the parental strains. On the other hand, Pham and Halos (1990) used intergeneric protoplast fusion between *T. reesei* and *P. funiculosum* to improve cellulose production. They obtained some fusants, which exhibited improved characteristics for cellulose activity when compared to either of their parents. Talkhan (2000) used mutation induction and protoplast fusion technique for improving α-amylase production in *Aspergillus foteidus* and selected some mutants, which over-yielded their original strain in the enzyme production. She obtained 18 stable fusants, after three different protoplast combinations between three mutants. Few fusants over-yielded a higher parent in the capacity of enzyme production, whereas most of them produced the enzyme within their parental range. This indicated utilization of a high enzyme productive mutant as a parent in protoplast fusion led to high productive fusants.

The present study was aimed to improve the

To cite this paper: Talkhan, F.N., H.G. El-Masry, S.M. Hassanein[1], F.M. El-Beih and M.S. Abdel-Aziz. 2008, Genetic improvement of dextranase production by *Penicillium funiculosum* via mutation induction and protoplast fusion. *Int. J. Agri. Biol.,* 10: 42–46

production of the dextranase enzyme in a *P. funiculosum* strain upon induction of mutations and protoplast fusion technique.

MATERIALS AND METHODS

This study was carried out in the Applied Microbial Genetics Laboratory, Genetics and Cytology Department, National Research Centre, Cairo, Egypt.

The fungal strain *Penicillium funiculosum* NRRL 6014 was kindly provided by Northerm Regional Research Laboratory, Department of Agrie, U.S.A. and used for the induction of mutations by UV light. A Philips TUV-30 W, WL 254 mm, Lamp type number 57413 was used as the source of radiation treatments.

Media. The media used throughout this study were:

Complete medium (CM). This medium was used for growing the *Penicillium* strain, mutants derived and their obtained fusants. It is composed of g L^{-1}: glucose 30; peptone 5.0; $MgSO_4$ $7H_2O$ 0.5; KH_2PO_4 1.0; yeast extract 0.5 and agar 20 and distilled water 1 L.

Minimal medium (MM). (Macdonald *et al*., 1963) it was used for the isolation of auxotrophs. It is composed of the following components g L^{-1}: $NaNO_3$ 3.0; KCI 0.5; Mg $SO_4 7H_2O$ 0.5 g; Fe $SO_4 7H_2O$ 0.01; KH_2PO_4 1; glucose 40; agar 20 and 1.0 L, distilled water adjusted to pH 6.8.

Antifungal medium or selective medium. It contained the same components of the complete medium with the addition of one or more of antifungal agent. The used antibiotics were griseofulvin (100 µg mL^{-1}), cycloheximide (250 µg mL^{-1}) and nystatin (50 units mL^{-1}). These antifungal antibiotics were purchased from Sigma. The commercial fungicide, topsin (2.5 µg mL^{-1}) was also used in this study. All antibiotics were dissolved in dimethylsulfoide (DMSO) before adding to medium and filter-sterilized antibiotics were then added when required. All these media were autoclaved at 121°C for 20 min.

Enzymes. The Novozyme 234 (Sigma) was used in this study for preparing protoplasts. It is composed mainly of cellulase, chitanase and protease.

Ultraviolet irradiation (UV) treatments. Spore suspensions were prepared in saline solution (0.85% NaCI) from the slants 10 days old and irradiated for 0, 4, 6, 8, 10 and 15 min at a distance of 20 cm from the lamp. Treated spores were kept in dark for one hour before they were diluted and spreaded on to CM plates. These plates were incubated for 3 days at 29 ± 1°C and single colonies were then transplanted to CM slants.

Isolation and identification of auxotrophic mutants. Single colonies, which appeared on CM plates after mutagen treatment were tested on CM and MM plates and incubated for 3 days at 29°C. The isolates, which failed to grow on MM plates were considered as auxotrophs. In addition, all colonies, showing abnormal growth, colors and/or shapes were considered as morphological variants.

Formation of protoplasts. For each strain, 40 mL of CM in a 150 mL Erlenmeyer flasks were inoculated with a scarp of mycelium grown on CM slant and incubated on a rotary shaker (250 rpm) at 28°C for 48 h. Afterwards, flasks were harvested and the mycelia were recovered aseptically by centrifugation, then washed twice with distilled water and resuspended in NaCI 0.7M, pH 5.6 supplemented with 5 mg mL^{-1} Novozyme 234. The lytic mixture was incubated at 28°C with gentle shaking for up to 3 h. Protoplasts were detected microscopically, which could be separated from mycelial debris by slow centrifugation (Reymond & Fevre, 1986).

Protoplast fusion. Protoplasts were washed twice with 0.7 M NaCI. Equal numbers of protoplasts from the two parents were mixed and centrifuged for 10 min. The pellet was then resuspended in 1 mL of a pre-warmed (30°C) solution of 20% (W/V) polyethylene glycol 6000 (PEG) containing 0.01 M $CaCI_2$ and 0.05 M glycine buffer (pH 7.5) and incubated at 30°C for 10 min. Later, treated protoplasts were washed twice with 0.7 M NaCI and finally resuspended in 5 mL 0.7 M NaCI (Reymond & Fevre, 1986).

Isolation of fusants. Polyethylene glycol treated protoplast suspensions were plated on to an antifungal selective medium, which was partially supplemented with the antifungal, griseofulvin, cycloheximide, nystatin and topsin, when fused cultures showed different antifungal resistance degrees. Seeded plates were incubated at 30°C for 4 days. Colonies grown on the surface of the plates were considered as complemtary fusants. They were transplanted on to CM slants for further tests.

Determination of dextranase activity. Different mutant strains alone with the original strain were screened qualitatively on a solid medium containing blue dextran as an indicator of dextranase activity as reported by Galvez-Marshal and Lopez-Munguia (1991). The positive dextranase producing mutants show a chear zone around the culture. The positive strains were then selected for quantitative determination of the enzyme according to the method mentioned by Szczodrak *et al*. (1994).

RESULTS AND DISCUSSION

Different microorganisms have been reported for dextranase production in general, fungi are the most widely distributed micro-organisms with dextranolytic activity. The best producers of the extracellular dextranase enzyme are the species of *Penicillium*. The fungal strain *P. funiculosum* NRRL-6014 was used in this investigation as an original isolate for the induction of high dextranase activity mutants using UV irradiation.

Induction of mutation in *P. funiculosum*. Utilization of induced mutants played an essential role in improvement of most industrial fungi productivity for the important products such as enzymes, organic acids, antibiotics and others. The fungal strain *P. funiculosum* NRRL-6014 was treated with UV light in different doses (exposure periods), after which survival percentages were estimated as shown in (Table I).

Survival rate decreased with increased the UV exposure time, where survival was 6.80% after 4 min and decreased drastically to reach 3.27, 1.45, 1.09 and 0.87% after 6, 8, 10 and 15 min, respectively. This decrease in survival rate may be attributed to damages in nucleic acids or defects in other cell components caused by UV irradiation. Talkhan (2000) found that survival percentages in *Aspergillus foetidus* decreased gradually with the gradual increase in the exposure time of the fungal conidia to UV light. A survival rate of 4.6% to 27.5% after the treatment with UV irradiation for 2 and 4 min, respectively was mentioned for *P. notatum* by Szezodrak *et al*. (1994) as shown in (Table I). A total of 100 stable mutants were obtained, 15 of which after 4 min, 18 after 6 min, 20 after 8 min, 22 after 10 min and 25 after 15 min, giving mutation percentages of 11.71, 17.64, 19.04, 23.15 and 30.48%, respectively. Some of these mutants were chosen as morphological variants (Fig. 1), which differed from the original strain (in color, shape or growth vigor) others were selected as auxotrophs. These mutants proved to be the most stable isolates out of 512 colonies tested on MM and CM media. Induction of mutations in *Penicillium sp.* by UV light was reported by Agrawal *et al*. (1999), Steiner *et al*. (1998) and Gomarteli *et al*. (1998) as a tool for strain improvement. Hoffman and Wood (1985) obtained several mutations of *P. funiculosum* by UV-irradiation, which differed from the wild type strain. Brown *et al.* (1987) isolated a number of mutants from *P. pinophilum,* which showed quite differences in the morphology of the mycelium from that of the wild type.

Dextranase activity of *P. funiculosum* mutants. In order to choose the dextranase producers, a total of 100 fungal mutants were examined for extracellular dextranase activity according to the procedure mentioned by Galvez-Marshal and Lopez-Munguia (1991). Based on this screening procedure, 40 mutants showed wide-clear zones, indicating that these mutants had high enzyme activity, whereas the remaining 60 mutants showed either narrow clear zone or did not show any clear zone. The most active 40 mutants along with a non-producing one were subjected for accurate determination of enzyme production in comparison with their parental strain according to the method reported by Szczodrak *et al*. (1994).

Data revealed that original strain (wild type) gave 140.6 U mL^{-1} dextranase activity whereas 10 mutants produced less enzyme than the wild type strain (Table II). Thirty mutants produced the enzyme more than the wild type strain, six of which showed dextranase activity up to 2 fold or more than that obtained by the wild type strain. Mutant strain No. 10 produced 371.9 U mL^{-1} of the enzyme or 264.5% of the wild type production, and was considered as the highest enzyme producer (Table II). The mutant strain No. 2 produced 243.33 U mL^{-1} of the enzyme or 173.1% and the mutant No. 18 produced 269.21 U mL^{-1} or 191.5% of the wild type production, which are considered as moderate high enzyme producers.

The mutant strain No. 12 produced 292.53% U mL^{-1} or 208.1% of its parent and is considered as a high enzyme producer. The mutant strain No. 29, which is morphologically different from the other mutants and wild type strain, gave 176.24 U mL^{-1} or 125.3% of its parent activity and is considered as a moderate enzyme producer. The mutant strain No. 4, chosen as a non-enzyme producing one, showed no dextranase activity, proving that it lost its ability to produce this enzyme (Table II).

Improvement of fungal strains for more enzymes production through UV-induced mutations in *Penicillium* spp. was recorded by other investigators *P. funiculosum* for cellulose by Hofman and Wood (1985), *P. notatum* for dextranase by Szczodrak *et al*. (1994), in *Lipomyces starkeyi* for dextranase by Kim and Day (1994) and in *A. foetidus* for amylase by Talkhan (2000).

Protoplast fusion and dextranase production. From the induced mutants obtained after treatment of the original strain with UV light, only four mutants were selected for protoplast induction according to a) dextranase productivity, b) morphological variants, c) resistance or sensitivity to one or more of the four antifungal agents used. These mutant strains were No. 2, 12, 18 and 29 (Fig. 1 & Table III).

Table I. Survival ratio and mutation percentage of *P. funiculosum* NRRL-6014 obtained after UV-irradiation treatments

Treatment time (min)	Survival		Mutation		
	No. of colonies	%	No. of tested colonies	No. mutants	%
0	9484	100	200	0.0	0.0
4	645	6.80	128	15	11.71
6	311	3.27	102	18	17.65
8	138	1.45	104	20	19.04
10	104	1.09	95	22	23.15
15	85	0.87	82	25	30.48
Mean	256.6	2.69	102.4	20	20.40

Table II. Dextranase production by the induced mutants compared with the wild type strain

Mutant No.	Enzyme Activity		Mutant No.	Enzyme Activity	
	U mL^{-1}	% from W.T.		U mL^{-1}	% from W.T.
W.T.	140.6	100	31	258.11	183.6
1	255.34	181.6	32	190.11	135.2
2	243.33	173.1	33	249.79	177.6
4	0.00	0.00	34	277.54	197.4
6	293.50	208.7	35	174.85	124.4
7	293.50	208.7	36	212.32	151.1
10	371.9	264.5	37	277.54	197.4
11	209.54	149.1	38	262.28	186.5
12	292.53	208.1	40	291.42	207.3
13	158.20	112.5	41	131.83	93.8
14	208.78	148.5	44	66.61	47.4
15	259.78	184.8	48	130.44	92.8
17	223.42	158.9	50	127.67	90.8
18	269.21	191.5	53	98.53	70.1
22	210.24	149.5	60	162.78	115.8
23	163.75	116.5	64	77.71	55.3
24	245.62	174.6	67	181.79	129.3
27	115.18	81.9	71	122.12	86.9
28	244.24	173.7	76	97.14	69.1
29	176.24	125.3	78	151.26	107.6
30	334.44	237.8	81	128.77	98.7

Fig. 1. Morphological variants of the induced mutants compared with the wild type strain

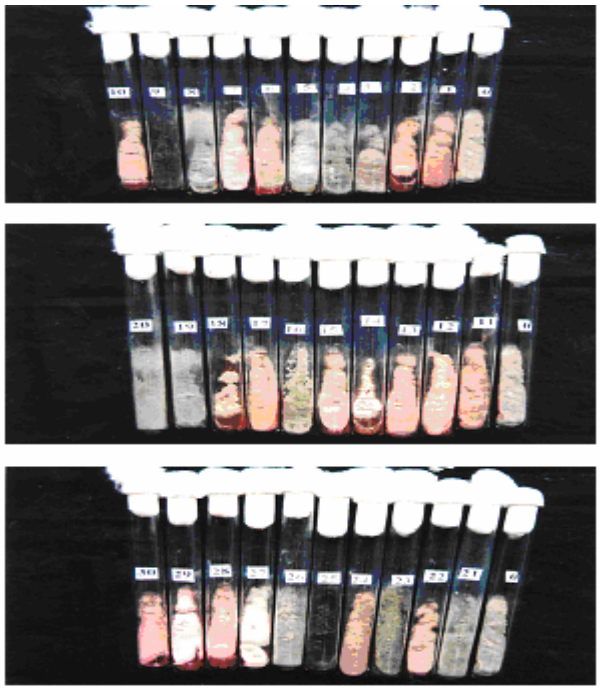
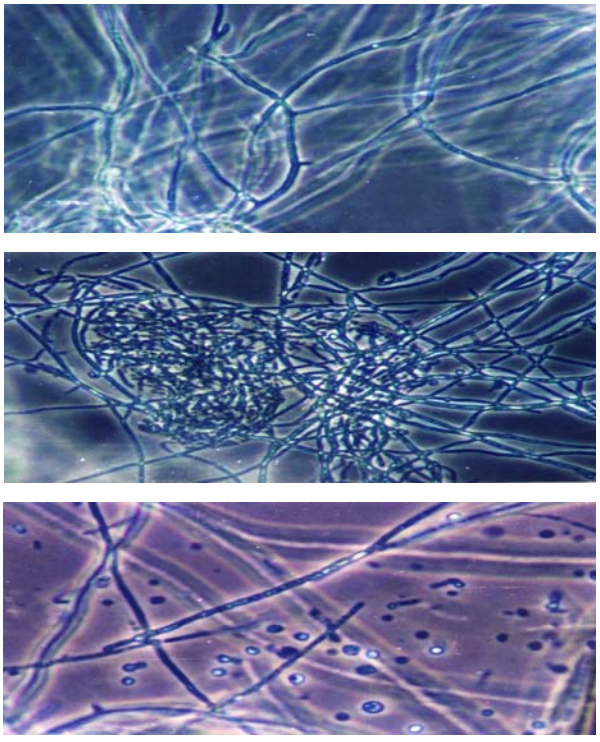


Fig. 2. Various stages of the protoplasts of mycelia of *P. funiculosum* mutants



Mutant No. 2 is characterized by its moderate high productivity (173.1% of the wild type), sensitive to griseofulvin and resistant to the other antifungal agents used. Mutant No. 12 is characterized by its high productivity (208.1% of the wild type), sensitive to griseofulvin and topsin but resistant to each of nystatin and cycloheximide. Mutant No. 18 is a moderate high productive (191.5% of the wild type), resistant to griseofulvin and sensitive to the other antifungal agents. Mutant No. 29 is a moderate productive (125.3%), sensitive to each of griseofulvin and topsin but resistant to nystatin and cycloheximide like the mutant No. 12. Thus, mutant No. 18 differed from the other three ones in the antifungal reactions and was used as a common parent in the three combinations of protoplasts fusions as follows:

(i). Mutant No. 2 (MH): mutant No. 18 (MH).
(ii). Mutant No. 12 (H): mutant No. 18 (MH).
(iii). Mutant No. 18 (MH): mutant No. 29 (M).

Protoplast fusion in fungi may yield some diploids or heterokaryons, which genetically varied from their original cultures. It is well known that fused protoplasts or fusants may produce stable diploid or heterokaryons, which genetically varied from their original cultures. It is well known that fused protoplasts or fusants may produce stable diploid recombinants from which certain desired genotypes can be selected as promising strains. In the present investigation, protoplast fusion was carried out aiming the isolation of new recombinants (fusants) with high capacity

Table III. Antigungal response and dextranase activity of chosen mutants for protoplast fusion and regeneration

Mutant No.	Exposure time (min)	Enzyme activity		Antifungal agents			
		U mL^{-1}	% of W.T.	Nystatin	Griseofulvin	Topsin	Cyclo-heximide
2	15	243.33	173.1	+		+	+
12	4	292.53	208.1	+			+
18	10	269.21	191.5		+		
29	4	176.24	125.3	+			+

Table IV. Dextranase activity of the fungal fusants resulted from the three combination of protoplast fusion in *Penicillium funiculosum* NRRL-6014 mutants

Fusants	Enzyme Activity		Fusants	Enzyme Activity	
	U mL^{-1}	% of W.T.		U mL^{-1}	% of W.T.
$2^{MH}x18^{MH}$			$12^{MH}x18^{MH}$		
1	208.16	148.52	20	467.65	333.65
2	270.61	193.07	21	492.63	351.48
3	237.30	169.31	22	355.46	253.61
4	256.72	183.16	23	453.78	323.76
5	259.50	185.15	24	403.82	288.10
6	252.56	180.19	25	469.04	334.65
7	277.55	198.02	26	441.29	314.85
8	249.79	178.22			
9	304.46	217.22	$18^{MH}x29^{M}$		
10	292.81	208.91	27	270.61	193.07
11	303.91	216.83	28	210.93	150.49
12	380.32	200.00	29	237.30	169.31
13	274.76	196.03	30	222.03	158.41
14	228.97	163.36	31	291.41	207.91
15	278.93	199.01	32	252.56	180.19
16	327.67	233.78	33	305.29	217.82
17	292.80	208.90	34	248.40	177.23
18	287.25	204.94	35	285.87	203.96
19	308.07	219.80	36	259.51	185.15

MH= moderate high productive. H = high productive. M = moderate productive

of dextranase production.

The stages of protoplast formation in mycelia of *P. funiculosum* mutants are shown in (Fig. 2). Upon protoplast fusion technique, a total of 19 stable fusants were obtained from the cross combination (a) which grew on the selective medium and proved to be resistant to all antifungal agents used. The cross combination (b) gave seven fusants, which grew on the selective medium and proved to be resistant to nystatin, griseofulvin and cycloheximide together. Ten stable fusants were selected from the selective medium used in the cross fusion (c) showing resistance to these three antifungal agents as cross (b) combination. These 36 fusants were tested for dextranase production compared with the productivity of the wild type strain NRRL-6014 and their enzyme activities are presented in (Table IV). Dextranase activity of all tested fusants exceeded that of the original strain by about 50% to 250%. Such results are expected since the parental mutants used for protoplast fusion combinations had enzyme activity more than the original strain by about 25% in the mutant No. 29 (M) to 108% in the mutant No. 12 (H) as shown in (Table III). Nevertheless, the cross combination between the high productive mutant No. 12 and the moderate high mutant No. 18 gave the most productive fusants in which enzyme activity over-yielded the wild type by about 154% (fusant No. 22) to about 251% (fusant No. 21) as shown in (Table IV). On the other hand, the fusion between this moderate high productive (mutant No. 18) and the moderate mutant No. 29 produced ten fusants in which enzyme activity exceeded that of the wild type by about 50% (fusant No. 28) to about 118% (fusant No. 33). The fusion combination between the two moderate high productive mutants No. 18 and No. 2 gave 19 fusants in which dextranase activity over-yielded the wild type by about 49% (fusant No. 1) to about 134% (fusant No. 16). These data concluded that protoplast fusion between high enzyme productive strains is expected to give higher enzyme productive fusants than those resulted from other crosses involving moderate or low enzyme activity ones.

Present results agree those of the other investigators (Halos *et al*., 1989; Talkhan, 2000), who reported that some formed fusants surpassed their parental strains in enzymes activity. Talkhan (2000) concluded that utilization of a high enzyme productive mutant as a parent in protoplast fusion gave high enzyme productive fusants.

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(Received 20 October 2006; Accepted 22 November 2006)