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# *Penicillium notatum* 1 a new source of dextranase

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## SUMMARY

Two hundred and fifteen fungal strains were screened for extracellular dextranase production with a diffusion plate method. The best enzymatic activity (12–19 DU $ml^{-1}$) was achieved by *Penicillium notatum* 1, a species for which the dextranase productivity has not yet been published. Some of the parameters affecting enzyme production have been standardized. The enzyme in crude state was relatively stable, its maximal activity was at 50 °C and at pH 5.0. Conidia of the selected strain were mutagenized, and isolated mutants were tested for production of dextranase in submerged culture. The most active mutant, *P. notatum* 1-I-77, showed over two-times higher dextranase activity than the parent *P. notatum* 1.

## INTRODUCTION

Dextranase ($\alpha$-1,6-glucanohydrolase, EC 3.2.1.11), which hydrolyses the $\alpha$-1,6-glycosidic linkages of dextran, has become a very useful enzyme with wide applications, especially in the medical field [4,5] and in the sugar refining industry [1,3].

Various cultures of moulds have been investigated as a source of dextranase [8,9,22,24], but no report has mentioned the utility of mutant strains to improve enzyme activity or methods for preliminary selection of mutants. A recent screen of mould species showed that strains of *Penicillium funiculosum, P. aculeatum* and *Chaetomium gracile* are producers of high levels of extracellular dextranase [10,13,16]. However, the search for additional dextranase producers among wild-type fungi is necessary to find potentially new sources with different characteristics. Therefore, the present study was conducted to select the best dextranase-producing fungus, to optimize the production of enzyme in submerged culture, and to intensify dextranase activity via mutagenesis.

## MATERIALS AND METHODS

### *Strains, media and screening procedures*

Two hundred and fifteen fungal cultures were tested, including representatives of the genera *Acremonium, Alternaria, Aspergillus, Botryotrichum, Botrytis, Chaetomium, Fusarium, Gliocladium, Humicola, Mortierella, Mucor, Myrothecium, Neurospora, Paecilomyces, Papularia, Penicillium, Phanerochaete, Rhizopus, Spicaria, Sporotrichum, Trichoderma, Trichothecium*, and *Verticillium*.

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The first selection of dextranase-producing fungi was done by plating conidia on medium A (pH 5.5) which contained (g $L^{-1}$): dextran (Mw, $40 \times 10^6$), 10.0; $KH_2PO_4$, 2.0; $NaNO_3$, 2.0; yeast extract, 0.5; agar, 20.0. After incubation (96 h at 30 °C), dextranase-active colonies were surrounded by a clear zone on an opalescent background (plate method a).

Isolates showing the greatest diffusion areas were examined more accurately by test tube cultivation (25 mm diameter, 12 ml medium B) on a rotary shaker at 220 r.p.m. The basal medium B for enzyme production (pH 5.5) consisted of (g $L^{-1}$): dextran (Mw, $110 \times 10^3$), 15.0; yeast extract, 2.0, and mineral salts as in medium A. After 96 h incubation at 30 °C, dextranase activity was determined. In this approach the spent medium (0.05 ml) was dropped into 8-mm diameter wells in a buffered (pH 5.0) agar (2%, plated in a 4-mm layer) containing 1% dextran (Mw, $40 \times 10^6$). The agar plates were incubated 18 h at 30 °C. Clear zones of hydrolyzed dextran were formed (plate method b). The diameter of the enzyme diffusion zone (in mm) was taken in both plate methods as the index of the extracellular dextranase.

Finally, the best dextranase producers selected on the basis of these criteria were incubated in 500-ml conical flasks containing 100 ml of basal medium B. The medium was seeded with conidia to a final concentration of about $2 \times 10^5$ conidia $ml^{-1}$. The submerged culture was run for 4 days (if not otherwise indicated) at 28 °C on a rotary shaker at 220 r.p.m. Then the mycelium was separated by filtration through Miracloth quick filtration material (Chicopee Mills, Inc., Milltown, NJ, USA) and the filtrate was used as enzyme solution for various tests.

### *Mutagenesis*

The best dextranase-producing strain was used as a starting culture for mutation. A conidial suspension ($10^7$

conidia $ml^{-1}$) was exposed to UV irradiation ($1.92 \times 10^3$ erg $mm^{-2}$) and treated for 10 or 20 min with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at a final concentration of 0.01%. To stop NTG activity, the conidia were separated from the mixture by centrifugation (5 min at $10000 \times g$), suspended in sterile distilled water and used as an inoculum for mutant selection. In another experiment, the conidia were irradiated only with UV at a dose of $3.84 \times 10^3$ erg $mm^{-2}$. The survival rate after the mutagenic treatment was 4.6–27.5%. The screening procedures were used to identify mutants with increased dextranase activity.

*Enzyme assay*

Dextranase activity was measured at 50 °C and at pH 5.0 in 0.1 M acetate buffer with 2% dextran (1 ml, Mw $110 \times 10^3$) and 0.5 ml of culture filtrate. After 30 min incubation, the liberated reducing sugars were monitored by the 3,5-dinitrosalicyclic acid (DNS) method [19]. One unit of enzyme (DU) was defined as that amount which produced one μmole of isomaltose (measured as maltose) per minute and was expressed as units per ml of culture (DU $ml^{-1}$). Specific activity was defined as dextranase units per milligram of protein. Protein was quantified by the method of Schacterle and Pollack [20]. Other methodological details are given in the Tables and Figures.

Fermentations were performed in triplicate, and analyses were carried out in duplicate. The data given are the means of all the measurements.

## RESULTS AND DISCUSSION

*Choice of dextranase producer*

A total of 215 fungal cultures belonging to 23 genera were examined for extracellular dextranase activity. Based on the screening procedure, seven strains were chosen, and their enzyme productivities in shaken flasks are presented in Table 1. The best dextranase producers comprised genera of *Penicillium* (except for the fungus *Acremonium humicola*). The highest dextranase activity per culture filtrate (14.9 DU $ml^{-1}$), as well as per mg of soluble protein (29.8 DU $mg^{-1}$), was reached by the strain *P. notatum* 1. Thus, further study was confined to this strain. All *Penicillium* species alkalized the medium slightly, resulting in a pH rise from 5.5 to above 7.0. The dry cell biomass ranged from 0.4 to 0.5 g per 100 ml.

*Optimization of dextranase production*

The relationship between the concentration of dextran and the amounts of enzyme produced is shown in Table 2. Maximal production of the enzyme occurred in medium containing 1.5% dextran. A concentration of 0.2% of each mineral salt and yeast extract, pH 5.5, and 100 ml of medium were adopted as the best conditions, which yielded about 14–16 units of dextranase per ml of culture broth after 4 days of cultivation.

Production of dextranase by *P. notatum* 1 was compared when the organism was grown in basal medium containing various carbohydrates as the sole carbon source. Dextran and insoluble dextran (Sephadex) were the most effective for enzyme production (Table 3). Unlike *Penicillium luteum* [7] and *P. aculeatum* [16,21] dextranases, the inducibility in *P. notatum* 1 is independent of the molecular weight of dextran. A small dextranolytic activity (0.2 to 1.8 DU $ml^{-1}$) was also found when glucose and maltose were carbon sources. Other carbohydrates had no effect on the enzyme induction.

Figure 1 illustrates a typical fermentation time course of *P. notatum* 1 in shaken flasks on the optimized basal medium B. Dextranase activity, extracellular protein, and mycelial biomass increased gradually and reached a maximum after 4 days of incubation. A 4-day period was chosen as the optimal time for production of dextranase by *P. notatum* in submerged culture. A study was also carried out to estimate

TABLE 1

Extracellular dextranase production by selected fungal strains after 4 days growth on basal medium B in shaken flask cultures

| Organism | Origin[a] | Dextranase activity | | Mycelial dry mass (g per 100 ml) | Final pH[b] |
|---|---|---|---|---|---|
| | | (DU $ml^{-1}$) | (DU $mg^{-1}$ protein) | | |
| *Penicillium lilacinum* 1 A | DAM | 7.2 | 9.0 | 0.4 | 7.3 |
| *Penicillium purpurogenum* 1 | DAM | 9.6 | 12.0 | 0.5 | 7.1 |
| *Penicillium janthinellum* 5A | DAM | 6.4 | 9.1 | 0.4 | 7.1 |
| *Penicillium notatum* 1 | IB | 14.9 | 29.8 | 0.5 | 7.2 |
| *Acremonium humicola* | DEM | 3.6 | 5.1 | 0.5 | 6.3 |
| *Penicillium vermiculatum* | DEM | 9.2 | 13.1 | 0.5 | 7.1 |
| *Penicillium funiculosum* F-161 | CCM | 3.2 | 6.4 | 0.5 | 7.3 |

[a] DAM, Department of Agricultural Microbiology, Agricultural University, Lublin, Poland; IB, Institute of Biotechnology, Warsaw, Poland; DEM, Department of Environmental Microbiology, University of Lublin, Lublin, Poland; CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic.

[b] Initial pH 5.5.

TABLE 2

Cultural factors affecting dextranase production by *P. notatum* 1 in basal medium B[a]: effect of pH, medium quantity, and some medium constituents

| Expt. no. | Factor varied | Concentration (%) | Dextranase activity[b] (DU $ml^{-1}$) |
|---|---|---|---|
| 1 | Dextran (Mw, 110 × $10^3$) | 0.5 | 4.9 |
| | | 1.0 | 6.7 |
| | | 1.5 | 14.1 |
| | | 2.0 | 9.4 |
| | | 3.0 | 7.6 |
| 2 | $KH_2PO_4$ | 0.1 | 10.3 |
| | | 0.2 | 14.5 |
| | | 0.4 | 13.0 |
| 3 | $NaNO_3$ | 0.1 | 9.2 |
| | | 0.2 | 14.2 |
| | | 0.4 | 11.1 |
| 4 | Yeast extract | 0.1 | 10.4 |
| | | 0.2 | 16.1 |
| | | 0.4 | 13.2 |
| 5 | pH | | |
| | 3.0 | | 8.9 |
| | 5.0 | | 11.8 |
| | 5.5 | | 13.6 |
| | 6.0 | | 12.3 |
| | 7.0 | | 12.1 |
| 6 | Volume of optimized medium taken (ml) | | |
| | 50 | | 9.0 |
| | 100 | | 15.7 |
| | 200 | | 11.5 |
| | 250 | | 6.8 |

[a] Composition of the medium was the same as that of the basal one, except that the factor or its concentration varied as indicated.

[b] Enzyme activity in culture filtrates was measured after 4 days of submerged cultivation in 500-ml conical flasks. The mean standard error of the enzyme estimate was ±0.27 and ranged from ±0.003 to ±0.72 DU $ml^{-1}$.

how much dextranase activity remained in the mycelium after fermentation. Only 7.1% of intracellular activity of enzyme was observed using mechanical disintegration with a Potter-Elvehjem homogenizer (Cole-Parmer Instrument Co., Chicago, IL, USA).

In further studies results with the optimized basal medium were compared with values in the literature for dextranase production in other media. After 4 days of cultivation our medium was 1.8-times more effective than the best medium cited in the literature (Table 4). Other media gave smaller or greater effects, and required longer periods of incubation (7 days). Moreover, the medium we propose for dextranase production has a simple chemical composition in comparison with complex media reported by other authors [11,15,21].

An attempt was also made to replace conidia of *P. notatum* by a suspension of its vegetative mycelium to shorten the cultivation time on basal medium. The use of 5% of the 48-h mycelium as inoculum shortened the incubation time by 24 h (Table 5), as compared to the use of the conidial suspension, maintaining dextranase activity on a level close (in case of specific activity even much higher) to that obtained in control runs. Shukla et al. [21] observed the highest formation of dextranase by *Penicillium aculeatum, P. purpurogenum*, and *P. funiculosum* with the use of an inoculum containing 4% of 48-h mycelium. Similar results were reported by Kosaric et al. [13] using a 5% suspension of *P. funiculosum* mycelium.

*Dextranase properties*

The crude enzyme (not stabilized) was stable for about two weeks at 0–4 °C and diluted enzyme (1:19, 0.1 M acetate buffer, pH 5.0) was also stable at room temperature for 24–48 h. It was found that the enzyme in crude state was also highly stable after two treatments – freezing and defrosting. Maximum dextranase activity was observed at pH 5.0 (Fig. 2), similar to dextranases from other microorganisms [5,10,12,13]. The enzyme was stable at pHs between 4.5 and 5.5 during storage at 30 °C for 24 h.

TABLE 3

Effect of carbon source on dextranase production by *P. notatum* 1

| Carbon source[a] | Concentration (%) | Dextranase activity (DU ml$^{-1}$) |
|---|---|---|
| Dextran (Mw, 4 × 10$^3$) | 1.5 | 10.4 |
| Dextran (Mw, 40 × 10$^3$) | 1.5 | 10.1 |
| Dextran (Mw, 110 × 10$^3$) | 1.5 | 11.9 |
| Dextran (Mw, 200 × 10$^3$) | 1.5 | 11.8 |
| Dextran (Mw, 500 × 10$^3$) | 1.5 | 11.8 |
| Dextran (Mw, 40 × 10$^6$) | 1.5 | 8.8 |
| Sephadex G-50 | 1.0 | 8.8 |
| Cellulose powder | 1.0 | 0.0 |
| Starch | 1.0 | 0.0 |
| Agar | 0.5 | 0.0 |
| Glucose | 1.0 | 0.2 |
| Fructose | 1.0 | 0.0 |
| Sucrose | 1.0 | 0.0 |
| Maltose | 1.0 | 1.8 |
| Lactose | 1.0 | 0.0 |

[a] The optimized basal medium B was used, except that dextran was replaced as indicated. After 4 days of cultivation, the dextranase activity of the culture filtrate was assayed by the standard method.

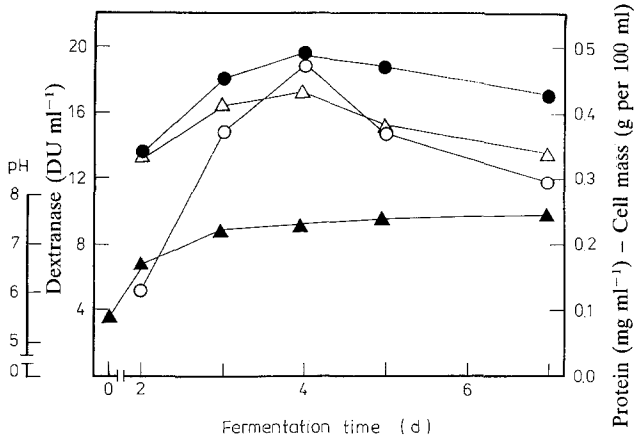


Fig. 1. Rates of growth, dextranase and protein production, and changes in pH during submerged culture of *P. notatum* 1 on basal medium B. -●- Protein; -○- Dextranase activity; -△- Cell mass; -▲- pH.

Dextranase from *P. notatum* 1 was most active at 50 °C (Fig. 3). Higher temperatures caused its rapid inactivation. Heat stability was examined at various temperatures for 10 min. Under these conditions, the enzyme was quite stable at temperatures up to 35 °C, but it was inactivated above 35 °C. At 50 °C a 60% fall in activity was observed. The enzyme was completely inactivated after 60 min at 50 °C. It should be stressed that the same dextranase showed a maximum activity at 50 °C in 30 min during its incubation in the presence of substrate. This was probably due to the dextran-protecting effect [18]. Blomhoff and Christensen [2] found that acetylated dextran increased thermostability of β-galactosidase from bovine testis.

TABLE 4

Effect of different media on dextranase production by *P. notatum* 1

| Medium | Days[a] | Dextranase activity | |
|---|---|---|---|
| | | (DU ml$^{-1}$) | (DU mg$^{-1}$ protein) |
| Proposed[b], pH 5.5 | 4 | 18.1 | 36.2 |
| Kosaric et al. [13], pH 5.8 | 7 | 12.5 | 17.9 |
| Hultin and Nordström [11], pH 5.0 | 4 | 9.9 | 49.5 |
| Shukla et al. [21], pH 5.5 | 4 | 10.2 | 25.5 |
| Lobanok et al. [15], pH 5.0 | 7 | 13.1 | 131.0 |
| Maksimov et al. [17], pH 5.5 | 7 | 4.6 | 23.0 |
| Fukumoto et al. [7], pH 6.0 | 4 | 8.8 | 18.7 |
| Tsuchiya et al. [24], pH 6.0 | 7 | 1.2 | 0.1 |

[a] Incubation period for maximum activity.
[b] Optimized basal medium B.

TABLE 5

Effect of the kind and amount of inoculum from *P. notatum* 1 on dextranase activity during submerged culture on basal medium B

| Inoculum quantity (v/v%) | Dextranase activity | | Medium pH |
|---|---|---|---|
| | (DU ml$^{-1}$) | (DU mg$^{-1}$ protein) | |
| 1[a] | 18.7 | 37.4 | 7.3 |
| 1[b] | 12.3 | 30.8 | 7.1 |
| 2[b] | 13.6 | 34.0 | 7.2 |
| 5[b] | 17.1 | 42.8 | 7.3 |
| 10[b] | 13.4 | 26.8 | 7.4 |
| 15[b] | 9.2 | 18.4 | 7.4 |
| 20[b] | 9.7 | 16.2 | 7.3 |

[a] Control inoculated with aqueous suspension of conidia of *P. notatum* (2 × 10$^5$ ml$^{-1}$); enzyme activity for the control test was determined after 4 days of culture.
[b] Experimental tests inoculated with a suspension of vegetative *P. notatum* mycelium (48 h) cultured on basal medium B with glucose (2%); enzyme activity was determined after 3 days of culture.

*Mutation studies*

In order to increase dextranase productivity, conidia of the best strain, *P. notatum* 1, were subjected to mutagenesis using UV or a combined UV-NTG technique. For preliminary selection of dextranase-overproducing mutants, the same screening procedures as those for the wild-type fungus were applied. Isolated clones were quantified for dextranase and protein yields in shake flask cultures on basal medium. The results for the best ten mutants are summarized in Table 6. The activity of dextranase of the investigated isolates in comparison with an initial nonmutagenized *P. notatum* 1

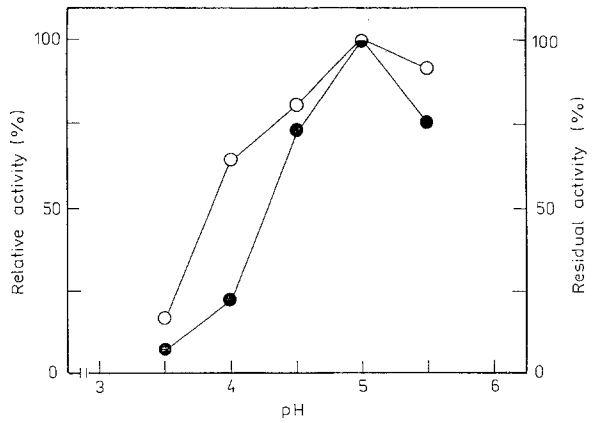


Fig. 2. Effect of pH on activity and stability of *P. notatum* 1 dextranase. –●– pH Activity curve using 0.1 M acetate buffer. –○– pH Stability curve. The stock culture filtrate was diluted 10-fold with 0.1 M acetate buffer of various pH values. After incubation at 30 °C for 24 h, aliquots were removed and the remaining activities assayed by the standard method.

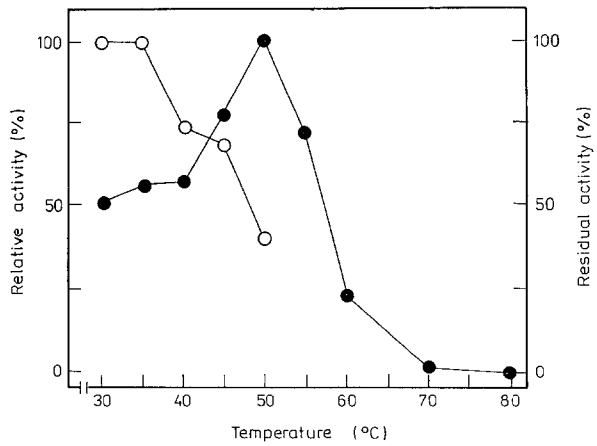


Fig. 3. Effect of temperature on activity and stability of *P. notatum* 1 dextranase. –●– Temperature activity curve. Activity was assayed by the standard method except that the reaction temperature was changed as indicated. –○– Thermal stability curve. The enzyme solution was incubated at various temperatures. After 10 min, it was cooled and residual activity was assayed by the standard method.

was generally higher (from 45% in case of *P. notatum* 1-II-24 to 131% in *P. notatum* 1-I-77). The increase in enzyme activity was not associated with a higher protein content in the culture medium. Therefore, we speculate that increased enzymatic activity may be caused either by an increased fraction of the native enzyme in the total protein secreted into the medium or by a change in the enzyme itself. The most active mutant, I-77, showed dextranase activity over twice as high (38.8 DU $ml^{-1}$) as the parent strain (16.8 DU $ml^{-1}$). The increased yield of dextranase production by the selected mutant was stable in subsequent subcultures.

Reports on mutagenic activation of fungal dextranase have not yet been published. Therefore, our results in this

TABLE 6

Dextranase and extracellular protein production by parent and mutant strains of *P. notatum* after 4 days submerged growth on basal medium B

| *P. notatum* strain | Mutagenic treatment (min) | | Survival rate (%) | Dextranase activity | | Extracellular protein (mg $ml^{-1}$) |
|---|---|---|---|---|---|---|
| | UV | NTG | | (DU $ml^{-1}$) | (%) | |
| Parent 1 | 0 | 0 | 100.0 | 16.8 | 100 | 0.6 |
| Mutant II-24 | 2 | 10 | 14.0 | 24.7 | 145 | 0.5 |
| IV-49 | 2 | 20 | 4.6 | 32.0 | 190 | 0.7 |
| IV-60 | 2 | 20 | 4.6 | 34.1 | 203 | 0.6 |
| IV-62 | 2 | 20 | 4.6 | 28.8 | 171 | 0.6 |
| IV-63 | 2 | 20 | 4.6 | 26.7 | 159 | 0.6 |
| IV-65 | 2 | 20 | 4.6 | 35.1 | 209 | 0.6 |
| IV-66 | 2 | 20 | 4.6 | 30.4 | 181 | 0.6 |
| IV-69 | 2 | 20 | 4.6 | 37.2 | 221 | 0.6 |
| I-73 | 4 | 0 | 27.5 | 26.0 | 155 | 0.5 |
| I-77 | 4 | 0 | 27.5 | 38.8 | 231 | 0.6 |

respect can be compared with data compiled by Kuek and Kidby [14], who improved glucoamylase production over 140% after mutagenesis of *Aspergillus phoenicis*. High results (over 125% increase of enzyme activity) were also reached in microbial synthesis of pectinolytic enzymes by *A. niger* [6]. Similar improvement related to cellulolytic enzymes was achieved by Szczodrak [23] after mutagenization of *Trichoderma reesei*.

In conclusion, the data presented show that the wild-type and the best mutant strains of *P. notatum* represent potentially new sources of extracellular dextranase and hence justify further investigations on application of this fungus in obtaining an active dextranolytic preparation.

## ACKNOWLEDGEMENTS

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