

Purification and Characterization of Dextranase From a New Strain of *Penicillium funiculosum*

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Abstract: Dextranase enzyme from a new mutant strain (M2) of *Penicillium funiculosum* NRRL 6014 was purified and characterized. The purification has been done using a sequence of chromatographic processes including ion exchange on Q Sepharose FF, gel filtration on Superose 12 and second ion exchange on Mono Q FPLC columns. Three active dextranase fractions (I, II & III) were produced. These fractions showed high purity and a molecular weights around 67 kDa as estimated by SDS/PAGE gel electrophoresis. The fractions had an optimum temperature of 55°C and thermal stability of 40°C. They also exhibited a pH optimum of 5.0 and pH stability over a pH range of 3-10. The enzyme fractions degraded dextrans with high specificity towards the α -1,6 glucosidic linkage. The effects of urea, guanidine hydrochloride and different native dextrans from different bacterial species on these enzyme fractions were investigated.

Key words: Dextranase, *Penicillium funiculosum*, mutation, purification, characterization

INTRODUCTION

Dextranases (α -1,6-D-glucan – 6-glucanohydrolase, EC. 3. 2. 1. 11) catalyze the hydrolysis of the α - 1,6 glucosidic bond of the polysaccharide dextran. Dextranases have been isolated from bacteria^[1,2,3], yeast^[4,5] and fungi^[6,7,8]. There are two major classes of this enzyme i.e., endo and exodextranases. Exodextranases release either glucose^[9,10] or isomaltose^[11] from the nonreducing ends of dextran and this has been identified in *Streptococcus mutans* and *Arthrobacter globiformis* T6, respectively. Most dextranases hydrolyze dextran in an endolytic fashion. The common endodextranases produce mainly both isomaltose and isomaltotriose from dextran as the final reaction products. The fungus *Chaetomium gracile* produces an endodextranase which hydrolyzes internal glucosidic bonds in dextran to produce oligosaccharides. Generally, dextranases from a variety of moulds and few bacteria are endotypes whereas exotypes are found predominantly in mammalian tissues^[12].

Dextranases are used in medicine for the preparation of blood substitutes by partial hydrolysis of dextran^[13]. Because dextran is involved in dental plaque formation, so dextranases are used in the manufacture of dentifrices as an additive for prevention of dental caries^[14]. This enzyme is also used in the characterization of a novel hydrogel with

polysaccharide-polyamino acids that used in the treatment of inflammatory bowel disease^[15]. Dextranases have been applied in sugarcane mills to diminish the levels of dextrans in deteriorated mill-juices with beneficial effects upon the processing rate^[16]. Dextranases can also be used in purification methods and studying glucose-containing polymers^[13,16]. Isomaltooligosaccharides was produced from *Penicillium lilacinum* dextranase immobilized onto EupergeticC^[17]. This oligosaccharide was also synthesized from sucrose in the presence of dextranasucrase and dextranase^[18]. Dextranasucrase was responsible for dextran formation and dextranase regulate the product molecular size. This work was aimed to Increase dextranase activity via genetic tools and use of different chromatographic methods to purify it.

MATERIALS AND METHODS

Organism and Media: A mutant strain of *Penicillium funiculosum* NRRL 6014, produced using UV irradiation treatment for 10 min, was maintained on a medium containing (g/l): glucose, 30; Peptone, 5; KH₂PO₄, 1; MgSO₄.7H₂O, 0.5; yeast extract, 0.5 and agar 15-20. The pH was adjusted to 6.0 and this is considered as a complete medium (CM) but the minimal medium (MM) has the following ingredients (g/l): NaNO₃, 3; KCl, 0.5; MgSO₄. 7H₂O, 0.5;

FeSO₄·7H₂O, 0.01; KH₂PO₄, 1; glucose, 40; agar, 20 and the pH was adjusted to 6.8^[19]. Moreover, the production medium had the following constituents (g/l)^[20]: Dextran, 15; NaNO₃, 2; MgSO₄·7 H₂O, 2.5 and yeast extract, 2. The pH of the culture medium was adjusted to 7. The strain was incubated at 30°C for 8 days on a rotary shaker (150 rpm). The cultures were centrifuged at 5,000 rpm for 20 min. The supernatant was then subjected to purification.

Mutagenesis: Mutation of wild type of the potent dextranase-producing fungus (*Penicillium funiculosum* NRRL 6014) was performed as follows: Spore suspension was prepared in saline solution (0.85% NaCl) from slant of 10 days old, and irradiated with UV light for 10 minutes at a distance of 20 cm. Treated spores were kept in dark for one hour before being diluted and spreaded onto the complete medium (CM). These plates were incubated for three days at 29°C and then single colonies were transplanted onto agar plates of both complete and minimal media.

Enzyme and Protein Assays: Dextranase was assayed by a modification of the method of Webb and Spencer-Martin^[21]. Enzyme preparations were incubated with 2.5% (wt/vol) Dextran T-500 (Pharmacia Inc., Piscataway, N.J.) in 0.05 M acetate buffer at pH 5.0 at 50°C for 10 min. Released reducing sugar were measured as glucose by the 3,5-dinitrosalicylic acid method^[22]. One unit (IU) of dextranase is defined as the amount of enzyme which liberates 1mmol of glucose equivalents in 1min. under the described conditions. Protein amounts were determined by the method of Lowry *et al.*^[23].

Dextranase Purification: The cell-free culture filtrate from M2 was clarified and applied to a series of purification steps including ion exchange on Q Sepharose FF column, gel filtration on Superose 12 column and ion exchange on Mono Q FPLC column. These chromatographic processes were carried on ÄKTA FPLC (Amershampharmacia biotech).

Enzyme Characterization: SDS-PAGE on PhastGel 8-25 electrophoresis with the use of low molecular weight calibration protein kit was used for both the determination of purity and molecular weight estimation. The optimum pH, pH stability, the optimum temperature and the thermal stability were measured. On the other hand, different substrates (dextran, xylan, starch, cellulose, DEAE-Sephadex, CM-Sephadex, glycogen, amylose, amylopectin, Sephadex (G10, 15, 25, 50, 75, 150 and 200), urea, guanidine hydrochloride and different native dextrans were studied for their effect on the enzyme activity.

RESULTS AND DISCUSSIONS

Mutagenesis: After UV irradiation the single colonies that appears on the CM were replica plated on CM and MM plate. Isolates which failed to grow on the MM plates were considered as auxotrophs. The colonies which showed abnormality in growth, color and shape were considered as morphological variants. This mutant showed a variation in both morphology and dextranase activity in relation to the wild strain. The induced mutant strain named M2 has been chosen because it had a dextranase activity of 265.89 U/ml (189.11% of the wild type). This result is analogous to that obtained from *Penicillium funiculosum* when it was used for the saccharification of rice straw^[24]. Mutagenesis had been used for increasing microbial dextranase enzymes from *Penicillium notatum* 1^[25] and *Lipomyces starkeyi*^[26].

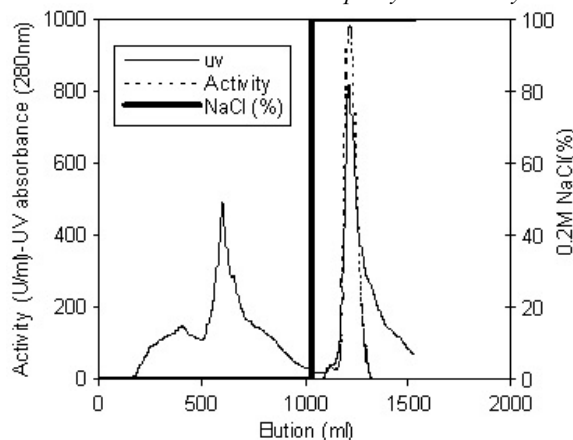


Fig. 1: Ion exchange chromatography on Q Sepharose (Vt 225 ml) fast flow. 500 ml of the culture filtrate from *Penicillium funiculosum* NRRL 6014 (M2) was applied to the column. The column was equilibrated with 20 mM of Bis-Tris (pH 4.8) and the elution was achieved by applying NaCl gradient 0.2 M (100%) at a linear flow rate of 10 ml/min.

Purification: Dextranase from mutant M2 showed a maximum dextranase active peak (961.39 U/ml) at an elution volume of 814.62 ml with a conductivity maximum of 17.72 mS/cm (100% of 0.2M NaCl) when applied to Q Sepharose FF (225 ml) column (Fig.1). Dextranase active peak from *Penicillium funiculosum* NRRL 6014 wild strain had the elution volume of 728 ml and conductivity maximum of 17.39 mS/cm. This dextranase active peak was collected, concentrated and applied to Superose 12 prep grade column and also one active peak was produced with an elution volume of about 880ml. (Fig. 2) and an activity of 1115.38 U/ml. Finally, this peak was collected and reapplied to Mono Q FPLC (Vt 10 ml) column giving three active dextranase fraction having conductivity maximum of 5.75, 6.20 and 7.07 mS/cm (38.1, 44.1, and 52.6 % of 0.2M NaCl) (Fig. 3). Abdel-Aziz^[20]

Table 1: The purification procedure of dextranase from *Penicillium funiculosum* (mutant strain M2).

Purification step	Total activity (U)	Total protein (mg)	Specific activity(U/mg protein)	Yield	Purification fold
Culture filtrate	101250.00	49.00	2066.33	100.00	1.00
Q Sepharose FF	68310.20	3.88	17605.67	67.47	8.52
Gel filtration	27565.85	1.43	19689.89	27.23	9.53
Mono Q FPLC					
I	3763.50	0.06	62725.00	3.70	30.36
II	16378.05	0.185	88530.00	16.18	42.84
III	4304.78	0.096	44841.68	4.25	21.70

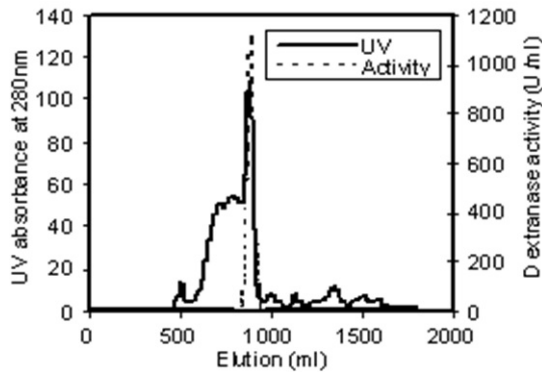


Fig. 2: Size-exclusion chromatography on Superose 12 prep grade 60/600. A concentrate (25 ml) of the dextranase active material from Q Sepharose FF (M2) was loaded to 6 60 cm Superose 12 prep.grade column (Vt 1700ml) equilibrated in 20 mM phosphate buffer pH 7.0 containing 0.2 M NaCl. The linear flow rate is 5 ml/min.

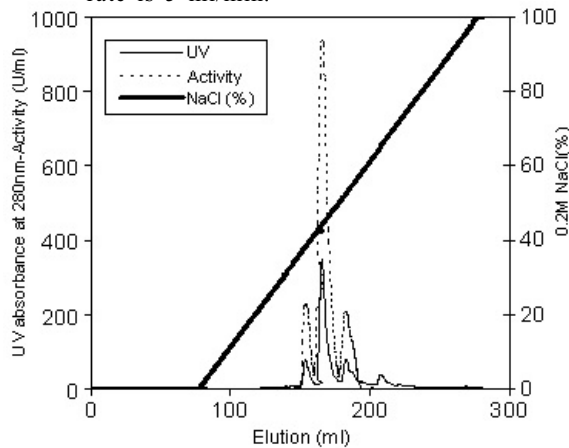


Fig. 3: Ion exchange chromatography on Mono Q FPLC. Sample of 25ml of crude dextranase from Superose 12 (M2) then used after concentration and desalting was loaded to a 1/10 cm Mono Q FPLC column (Vt 10ml) equilibrated in 20mM Bis-Tris, pH 4.8, containing 0.1 M NaCl. Linear flow rate: 60 ml/h.



Fig. 4: SDS-PAGE electrophoresis of different purification steps of *Penicillium funiculosum* NRRL 6014 (M2). Culture filtrate, ion exchange on Q sepharose FF and gel filtration on Superose 12 prep. grade 6/60 were analyzed by SDS/PAGE on Phast Gel gradient 8-25. Lane 1 and 8 represent the low molecular weight calibration protein, lane 2, 3, and 4 represent the culture filtrate, Q Sepharose and gel filtration step on superose lane 5, 6, and 7 represent the three dextranase fractions after Mono Q step.

found that two dextranase fractions were produced from wild strain of *Penicillium funiculosum* NRRL 6014 and four fraction from a fusant strain of the same fungus. Also, two pure dextranase fractions were produced from *Chaetomium gracile*^[27].

The Purification Table of Dextranase from the Mutant Strain M2: Table I summarizes all the purification steps of M2 strain of *Penicillium*

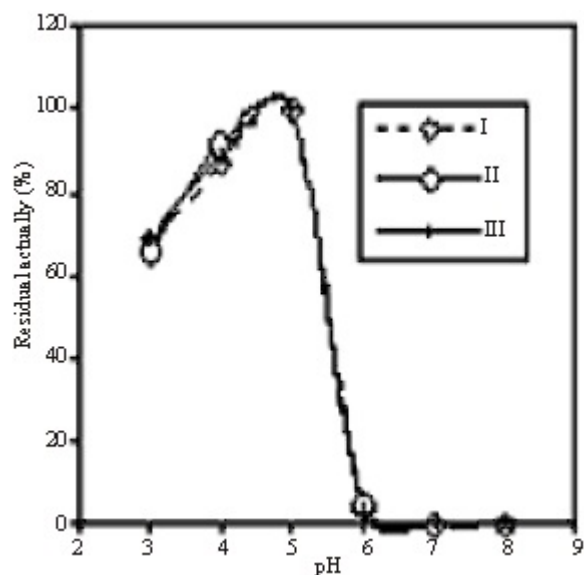


Fig. 5: pH value of different purified dextranase fractions from *Penicillium funiculosum* NRRL 6014 (M2)

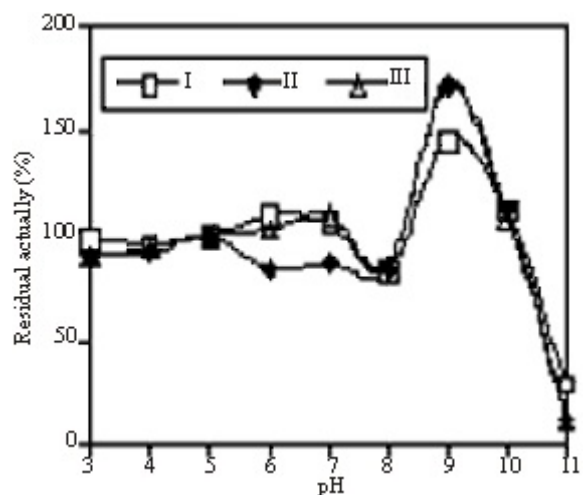


Fig. 6: pH stability of different purified dextranase fractions from *Penicillium funiculosum* NRRL 6014 (M2) after 1h.

funiculosum NRRL 6014. The total activity, total protein, specific activity, yield and the purification folds were calculated for M2. It was found that the purification folds of M2 were 30.36, 42.84 and 21.70 with a yield 30.36, 42.84 and 21.70% for fractions I, II and III respectively. Lee and Fox^[28] purified two dextranase enzymes (I&II) through two stage ion exchange chromatography, enzyme I showed a purification fold of 35.5 and 16% yield but dextranase II had purification fold of 19 and 4% yield.

Table 2: effect of different substrates on the different purified dextranase fractions of *Penicillium funiculosum* NRRL 6014 (M2).

Substrate	Main linkage	Residual activity of M2 (%)		
		I	II	III
Amylose	α -1,4	00.00	00.00	00.00
Amylopectin	α -1,4	00.00	00.00	00.00
Glycogen	α -1,4	00.00	00.00	00.00
Starch	α -1,4	00.00	00.00	00.00
Cellulose	β -1,4	00.00	00.00	00.00
Xylan	α -1,3	00.00	00.00	00.00
Dextrin	α -1,6	00.00	00.00	00.00
DEAE-Sephadex	α -1,6	28.13	22.31	29.57
CM-Sephadex	α -1,6	00.00	00.00	00.00
Sephadex G10	α -1,6	00.00	00.00	00.00
Sephadex G15	α -1,6	00.00	00.00	00.00
Sephadex G25	α -1,6	00.00	00.00	00.00
Sephadex G50	α -1,6	00.00	00.00	00.00
Sephadex G75	α -1,6	34.36	38.12	36.71
Sephadex G150	α -1,6	60.28	58.21	56.14
Sephadex G200	α -1,6	82.18	80.82	78.29

SDS/PAGE Gel Electrophoresis: The purified fractions produced from M2 of *Penicillium funiculosum* NRRL 6014 were examined for both purification degree and molecular weights using SDS/PAGE on Phast Gel gradient 8-25. Results showed the different purification steps, including culture filtrate, Q Sepharose, Superose 12 and Mono Q (Fig. 4). It was found that the produced dextranase fractions showed high degree of purity and molecular weight of about 67,000 Da.

Optimum pH and Stability: Optimum pH of the purified dextranase fractions from M2 were studied. It was found that the all fractions were more active at pH optimum of 5.0 (Fig. 5). On the other hand, all the fractions had the same pH stability profile and all fractions were stable up to pH 10 and decreased sharply at pH 11 (Fig. 6). The optimum pH and pH stability were found to be differ with the microorganism used. Dextranase from *Penicillium luteum* showed optimum pH between 4 and 6 with an optimum of 5.0 and had pH stability over a range of 3.5-7.0^[29]. Also, *Aspergillus carneus* dextranases were most active at pH values of 5.0-11.0 and were stable over a pH range from 4.5 to 9.0^[30]. Szczodrak *et al.*^[25] investigated that purified dextranase from *Penicillium notatum* 1 had an optimum pH of 5.0 and stability range of 4.5-5.5. It has also been found that the enzyme from *Fusarium* sp. exhibited pH optimum of 6.5 and was stable over a pH range of 4.5-11.8^[31].

Temperature Optimum and Thermal Stability: Results illustrated in Fig. (7) indicated that the fractions have the same temperature optimum of 55°C. The thermal stability was also studied and the results

Table 3: Effect of dextranase fractions (I, II, and III) from *Penicillium funiculosum* NRRL 6014 (M2) on different native dextrans.

Dextran	Dextran linkage (%)			Residual activity of M2 (%)		
	1,6	1,4	1,3	I	II	III
Dextran T500 (Control)	95	5	0	100.00	100.00	100.00
Dextran B- 512F	95	5	0	98.03	101.18	96.52
Dextran B- 641	94	3	3	94.81	95.7	96.34
Dextran B- 1400	93	3	4	96.29	101	94.18
Dextran B- 1392	91	6	3	102.73	97.78	96.07
Dextran B- 1225	90	10	0	81.19	82.38	79.95
Dextran B- 1255	89	7	4	68.84	75.6	74.73
Dextran B- 1144	87	7	6	72.83	74.95	74.95
Dextran B- 1351	85	4	11	71.46	69.49	73.13
Dextran B- 1389	85	7	8	83.79	83.04	85.78
Dextran B- 1443	80	10	10	86.77	57.07	61.97
Dextran B- 1399	65	35	0	77.785	65.34	69.74
Dextran B- 1298	64	36	0	18.52	18.47	16.53
Dextran B- 1355	57	8	35	00.00	00.00	00.00
Dextran B- 1299	50	50	0	00.00	00.00	00.00
Dextran B- 742	67	21	12	00.00	00.00	00.00

Linkage type percent according to Jeanes *et al.*(37).

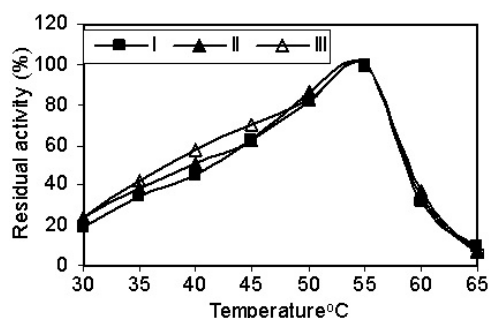


Fig. 7: Determination of the optimum incubation temperature of different purified dextranase fractions from *Penicillium funiculosum* NRRL 6014 (M2).

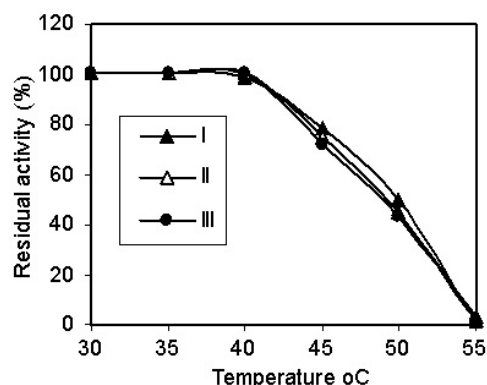


Fig. 8: Thermal stability of different purified dextranase fractions from *Penicillium funiculosum* NRRL 6014 (M2) after 30 min.

in Fig. (8) revealed that the dextranase fractions from M2 had the same profile of thermal stability and they were stable up to 40°C and the enzyme activity was sharply decreased by rizing the incubation temperature over 55°C. Madhu and Prabhu^[32] investigated that dextranase from *Penicillium aculeatum* had an activity maximum between 50 and 60°C and stable at 40°C. Also, Szczodrak *et al.*^[25] found that the optimum temperature of dextranase from *Penicillium notatum* 1 was 60°C and the enzyme was stable up to 35°C. On

the other hand, dextranase from *Penicillium funiculosum* showed a thermal stability up to 50°C^[33].

Influence of Urea and Guanidine Hydrochloride:

Fig. (9) exhibited the effect of different concentrations of urea and guanidine hydrochloride on dextranase fractions from the mutant strain M2 of *Penicillium funiculosum* NRRL 6014. It was found that urea had a

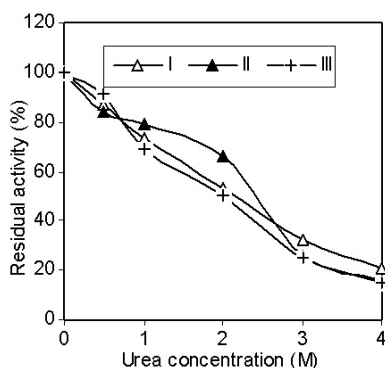


Fig. 9: Effect of different concentrations of urea on dextranase activity from *Penicillium funiculosum* NRRL 6014 (M2).

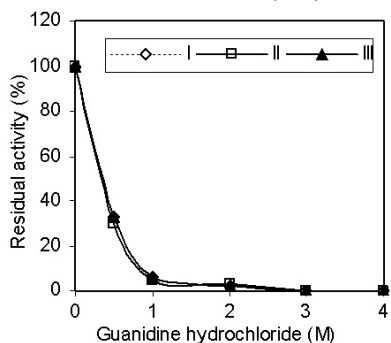


Fig. 10: Effect of different concentrations of guanidine hydrochloride on dextranase activity from *Penicillium funiculosum* NRRL 6014 (M2).

little effect at concentration of 0.5 and 1.0 M but a moderate effect has been shown in concentration of 2.0 M. On the other hand a sharp decrease in the activity has been found with urea concentration of 3.0 and 4.0 M. Otherwise, guanidine hydrochloride indicated a stronger effect than urea (Fig. 10). At a guanidine hydrochloride concentration of 0.5 M an activity loss of 60% was observed for all dextranase fractions and more than 90% activity loss has been observed with guanidine hydrochloride concentrations of 2.0, 3.0 and 4.0 M. The results were approximately analogous to that deduced by Janson^[34].

Substrate Specificity: The data presented in Table (2) revealed the effect of different substrates on the activity of different purified dextranase fractions from *Penicillium funiculosum* NRRL 6014 mutant M2. It was found that these enzymes showed an activity towards α -1,6 glucosidic linkage including dextran and dextran derivatives (Sephadex G 75, 150 & 200) but not with other carbons and highly cross-linked dextrans (Sephadex G 10-50). These results are in agreement

with that found by Fukumoto *et al.*^[29]. They reported that *Penicillium luteum* dextranase splitting preferentially a series of isomaltodextrins and dextran giving rise to glucose, isomaltose and a small amount

of isomaltotriose. They also found that Sephadex and its derivatives are also hydrolyzed. The enzyme was completely inert towards glucose polymers such as mannose, pullulan, isomaltosylmaltose, b-limit dextrin and waxy maize. The substrate specificity was also tested for dextranase from *Aspergillus carneus*^[35]. It was found that the enzyme could hydrolyze dextran T2000 to high degree and dextran IAM (66% α -1,6 glucosidic linkage) was slowly degraded.

Effect of Different Native Dextrans: Results in Table (3) showed the percentage of different linkage types affect dextranase activity. It was found that the degradation of dextran differ with the change in the percent of 1-6 glucosidic linkage. The activity was found to be decreased with decreasing this linkage percent and vice versa. Dextran NRRL 742 (75% (α 1,6) glucosidic linkage) when used as substrate for dextranase from *Penicillium funiculosum* NRRL 1768 exhibited a lower activity^[36] in relation to dextran B512f (95% of the same linkage). Tsuru *et al.*^[35] investigated that dextranase activity from *Aspergillus niger* had a very slow activity with dextran IAM (66% linkage).

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