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Cloning of a Gene Encoding Dextranase from *Lipomyces starkeyi* and its Expression in *Pichia pastoris*

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A gene (*lsd1*) encoding dextranase from *Lipomyces starkeyi* KSM22 has been previously cloned, sequenced, and expressed in *Saccharomyces cerevisiae*. The gene consisting of 1,824 base pairs and encoding a protein of 608 amino acids was then cloned into and secretively expressed in *Pichia pastoris* under the control of the *AOX1* promoter. The dextranase productivity of the *P. pastoris* transformant (pPIC9K-LSD1, 134,000 U/l) was approximately 4.2-fold higher than that of the *S. cerevisiae* transformant (pYLSD1, 32,000 U/l) cultured in an 8-l fermentor. Over 0.63 g/l of active dextranase was secreted into the medium after methanol induction. The dextranase of the *P. pastoris* transformant, as analyzed by SDS-PAGE and Western blotting, showed only one homogeneous band. This dextranase of the *P. pastoris* transformant showed a broad band near 73 kDa. Rabbit monoclonal antibodies against a synthetic LSD1 peptide mix also recognized approximately 73 kDa.

Keyworlds: Dextranase, *Lipomyces starkeyi*, *Pichia pastoris*, expression, dextran

In recent years, the methylotrophic yeast *Pichia pastoris* has been developed as a host organism for the expression of heterologous proteins [4]. Over 400 proteins from prokaryotes, eukaryotes, and viruses have now been successfully expressed in this yeast [3]. The *P. pastoris* expression system offers many advantages, including its ease of usage relative to other eukaryotic expression systems, the possibility of high-level expression of foreign proteins (either extracellularly or intracellularly), and its ability to undertake many eukaryotic post-translational modifications (e.g., glycosylation and proteolytic processing).

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Dextran is an α-D-glucopyranose homoglycan. It is synthesized by oral streptococci, which perform important functions in the formation of dental plaque [15]. It is also synthesized by the soil bacterium *Leuconostoc mesenteroides*, which infects sugarcane. This infection results in one of the primary problems in sugar production, as it affects yield by adulterating the purity of sucrose and increasing the juice viscosity. Dextranase (E.C. 3.2.1.11, α-1,6-glucan-6-glucanohydrolase) catalyzes the hydrolysis of the α-1,6-glucosidic linkages of dextran. There are two major classes of dextranases: exodextranases, which release either glucose or isomaltose from the nonreducing ends of dextran; and endodextranases, which hydrolyze internal glycosidic bonds in dextran, thereby generating oligosaccharides.

Commercial dextranase is produced by fermentation with either *Penicillium* sp. or *Chaetomium* sp. However, since these fungi also produce various antibiotics and toxic metabolites in addition to dextranase, this creates difficulty in obtaining FDA approval. The only yeasts reported to produce dextranases are members of the Lipomycetaceae family, and only *L. kononenkoae* [27] and *L. starkeyi* dextranases have been characterized [11, 12]. *L. starkeyi*, an ascosporogenous yeast, produces an endodextranase that cleaves the D-glucopyranosyl linkages in dextran, and an α-amylase (E.C. 3.2.1.1) that hydrolyzes the D-glucopyranosyl linkages in starch [10]. The dextranase of *L. starkeyi* ATCC 20825, a partial derepressed mutant, was studied by Koenig and Day [14]. The purified dextranase showed four bands with molecular masses of 65 kDa, 68 kDa, 71 kDa, and 78 kDa based on SDS-PAGE analysis, and apparently occurred as multiple isoelectric species, with pIs of 5.61, 5.73, 5.80, 5.95, and 6.06. Except for a few bacterial dextranases, microbial dextranases generally are inducible [1, 13]. Kim and Day [11] reported on the development of a derepressed and partially constitutive mutant (ATCC 74054) for dextranase and amylase, and

described the characterization of its enzyme. Ryu *et al.* [23] also reported on the purification and characterization of a novel glucanhydrolase from *L. starkeyi* KSM22 that exhibited either dextranase or amylase activity. This glucanhydrolase was a single, approximately 100 kDa protein based on SDS-PAGE analysis. The optimal pH and temperature for this *L. starkeyi* KSM22 glucanhydrolase were 5.5 and 37°C, respectively. Competition studies, employing different amounts of dextran and starch as substrates, yielded competition plots consistent with the hypothesis that dextran and starch hydrolysis occur at two independent active sites, each specific for starch or dextran [18].

The cloning and expression of a yeast dextranase gene in *P. pastoris* would allow for the generation of large amounts of enzyme that would be of potential utility in industrial fields. Extracellular dextranase from *L. starkeyi* has already been purified and partially characterized, and we previously isolated the cDNA encoding this enzyme by RT-PCR [9]. The present paper discusses the cloning and expression of the dextranase from *L. starkeyi* KSM22 in *P. pastoris*. Furthermore, some properties of the dextranase were also determined.

MATERIALS AND METHODS

Strain and Plasmid

The bacterial strains and plasmid used in this study are shown in Table 1.

Construction of the *P. pastoris* Expression Vector pPLSD1 (pPIC9K-LSD1)

The DNA fragment encoding the mature LSD1 protein was amplified by the polymerase chain reaction (PCR) from the pYLSD1 plasmid [9], using *Taq* DNA polymerase and the synthetic oligonucleotide primers LSD-EcoRIF (5'-ACGTTGTTGATTGAATTCATGACAT-3'), and LSD-NotIR (5'-CTTCCTTCGCGGCCGCCGA-3'). DNA amplification was conducted with 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 52°C), and elongation (2 min at 72°C). After PCR, the PCR product was digested with EcoRI (underlined at LSD-EcoR1F) and NotI (underlined at LSD-NotR), and the PCR fragment was then ligated into the pPIC9K vector (Invitogen, U.S.A.), resulting in the generation of plasmid pPIC9K-LSD1. All DNA manipulations were carried out according to standard procedures [22].

Transformation of *P. pastoris* and the Selection of Dextranase-producing Clones

Approximately 1 μg of pPLSD1 DNA was used for the transformation into *P. pastoris* GS115, *via* electroporation. Electroporation was essentially performed according to the method of Xu *et al.* [26], with cells pulsed in 0.2-cm electroporation cuvettes at 1.5 KV using a BioRad MicroPulser with Controller. One mL of cold 1 M sorbitol was then added to the cuvettes immediately after pulsing, and the His^+ transformants were recovered on minimal dextrose (MD) agar (1.34% YNB, 4×10^{-5}% biotin, and 2% dextrose) plates [22]. The plates were incubated for 3–6 days at 30°C.

The transformed cells were spread on YPD plates containing 4 mg/ml of geneticin (G418, Invitrogen, U.S.A.). Multicopy transformants were selected on the basis of G418 resistance. Then, each single colony was transferred to minimal methanol (MM) agar plates with 0.5% blue dextran (Sigma Co., U.S.A.), and dextranase expression was induced with methanol in vapor phase by adding 0.5-ml portions of 100% methanol under the lids of inverted plates. The plates were incubated overnight at 30°C and dextran digestion was indicated by the appearance of halos surrounding the colonies.

Five transformants that exhibited activity in the blue dextran plates were selected for dextranase expression studies. Cultures were grown in 100 ml of minimal glycerol medium (MGY: 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol) for 22 h at 30°C. The cells were then pelleted and resuspended in the same medium, but containing 1% methanol instead of glycerol, to induce the *AOX1* promoter (*AOX1*p), and then further incubated at 30°C. Additional 0.5% methanol pulses were supplied every 24 h during the culture growth for a total of 80 h. The dextranase activity in the culture supernatant was determined, and the strain designated as PLSD1-2 was selected for further fermentation studies.

Fermentation of *P. pastoris* Strain PLSD1-2

The fermentation studies with the PLSD1-2 strain were performed in a 10-l laboratory-scale fermentor (BioTron, Korea; 8 l working volume). The fermentor was inoculated up to OD_{600} at 0.2 and the

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or phenotype
Strain	
E. coli TOP10F'	F' {*pro*AB, *lac*I^q, *lac*ZΔM15, Tn*10*(Tet^R)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80 *lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str^R), *end*A1, *nup*G λ-
P. pastoris GS115	*his4* mutant
PLSD1-2	LSD1 production (this study)
L. starkeyi KSM	Dextranase production
Plasmid	
pGEM-T easy vector	Amp^r
pPIC9K	Amp^r, *HIS4*
pYLSD1	LSD1 in pYES2 (LSD1 production in *S. cerevisiae*)
pPIC9K-LSD1	LSD1 in pPIC9K

culture was grown in a chemically defined growth medium consisting of 30 g glycerol, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 14.3 g KOH, 7.0 g K_2HPO_4, 22.7 ml H_3PO_4, and 4.35 ml PTM1 trace solution per 1 l. Each liter of PTM1 trace solution consists of 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin, and 5 ml sulfuric acid [25]. The pH was adjusted to 5.0 with NH_4OH (28%, w/v). NH_4OH was used as a pH-control agent and nitrogen source. PTM1 trace salts were added after sterilization [19]. The fermentor was operated at 28°C, pH 5.0, 500 rpm, and an aeration rate of 1 vvm. Upon depletion of the glycerol (24 h after inoculation), the *AOX1* promoter was induced by adding 0.5% methanol. When the culture began to acidify, the methanol feeding rate was started at 2 g/l per hour, and the induction phase was prolonged for 120 h with a gradual increase in the methanol feeding rate up to 3.5 g/l per hour. The yeast cells were then separated from the broth by centrifugation. The pellet was discarded and the supernatant was dialyzed against 20 mM citrate-phosphate buffer (pH 5.2). The samples were tested for enzymatic activity and analyzed by SDS-PAGE and Western blot. The culture supernatant of *P. pastoris* strain without pPLSD1 did not produce dextranase.

SDS-PAGE and Western Blot Analysis

SDS-PAGE was performed as described by Laemmli [16] and Jung *et al.* [8]. The protein was stained with Coomassie Brilliant Blue R-250. After SDS-PAGE, the proteins in the gel were transferred electrophoretically to PVDF membranes. Immunodetection was achieved by monoclonal antibody generated against a synthetic peptide mix (LSD1-1 and LSD1-2) by immunizing rabbits [LSD1-1 (66-78): C-AGNVRQSRKYSVH-NH_2; LSD1-2 (279-291): C-EDTSGNPGKLGSN-NH_2]. Anti-LSD1-1 and LSD1-2 antibodies were used at a 1:200 dilution, and peroxidase-conjugated anti-rabbit-IgG [conjugate of goat anti-rabbit-IgG with horseradish peroxidase (Amersham Pharmacia Biotech)] was used at a dilution of 1:1,500 (v/v). Following incubation with the antibody, the filter membranes were washed three times with Tris-buffered saline (TBS; 20 mM Tris-HCl buffer, pH 7.6, 137 mM NaCl containing 0.1% Tween 20), followed by two more washes with the same buffer. The antigen-antibody complex was detected with secondary antibody using an ECL Western blotting analysis system (Amersham Pharmacia Biotech) and exposed on Kodak X-Omat AR film (Kodak, Rochester, NY, U.S.A.) for 1 min at room temperature.

Protein and Enzyme Activity Assays

Protein content was determined by the method of Bradford [2] with crystalline bovine serum albumin (Sigma) as a standard. Dextranase activity was determined based on the increasing ratio of the reducing sugar concentration in the reaction with copper-bicinchoninate reagent, according to the method of Fox and Robyt [6]. One unit (1 U) of dextranase was defined as the amount of enzyme that liberated 1 μmol of isomaltose equivalents in 1 min from dextran T-2000 at 37°C.

The rate of hydrolysis was determined with 0.5% substrate (dextran T-2000, dextran from *L. mesenteroides* B-742CB, dextran from *L. mesenteroides* B-1299CB4, alternan, and pullulan).

Thin-Layer Chromatography (TLC)

The pattern of dextran hydrolysis by the recombinant dextranase was examined using TLC [12].

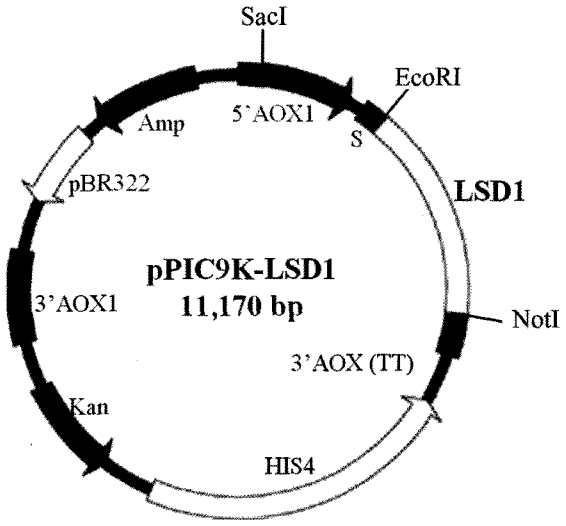

Fig. 1. The recombinant *Pichia* expression plasmid pPIC9K-LSD1 constructed for the dextranase gene from *Lipomyces starkeyi*.
The 5' *AOX1* promoter is a methanol-inducible promoter, S is the secretion signal sequence from *S. cerevisiae*, *and LSD1* represents the coding regions of the *Lipomyces starkeyi* dextranase gene. SacI is the restriction site used to linearize the plasmid before electroporating into *P. pastoris*.

Results and Discussion

Construction of Expression Vector and Expression of Dextranase in *P. pastoris*

The methylotrophic yeast *P. pastoris* and the integrating vector pPIC9K were selected as an expression system to produce a biologically active dextranase enzyme. The dextranase gene was amplified from *L. starkeyi* KSM22 (GenBank Accession No. AY280636) [9]. The PCR

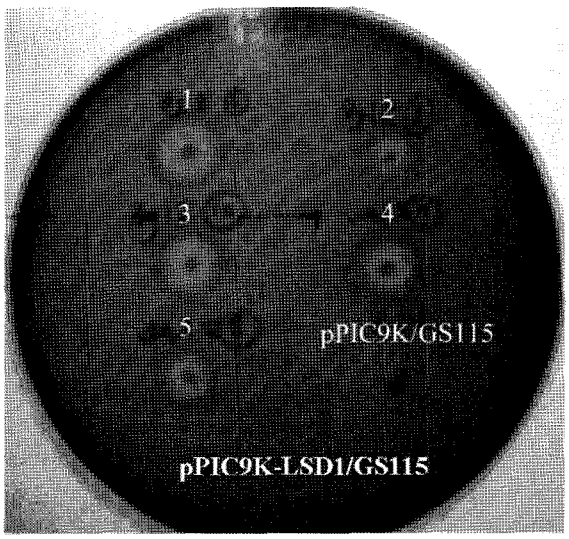

Fig. 2. Halos formed in a blue dextran agar plate by 5 transformants of *P. pastoris* producing dextranase enzyme.
The numbers 1 to 5 indicate the clones PLSD1-1 to PLSD1-5, respectively.

Table 2. Dextranase induction in 100-ml shake-flask cultures of 5 transformants of *P. pastoris*.

Clone	Final OD_{600}	Dextranase activity (U/l)[a]
PLSD1-1	19.7	1,860
PLSD1-2	20.4	2,740
PLSD1-3	18.6	1,780
PLSD1-4	21.3	2,263
PLSD1-5	19.7	2,050

[a]The level of secreted dextranase was quantified by measuring the enzymatic activity in the culture supernatant and checking the dextranase activity.

fragment encoding the mature LSD1 protein was cloned into the pPIC9K vector to yield the expression plasmid pPIC9K-LSD1 (Fig. 1). The pPIC9K-LSD1 plasmid was digested with SacI to generate a DNA fragment that was targeted for transplacement into the *AOX1* locus of *P. pastoris*. Transformation by electroporation of the GS115 (*his3*) strain with this DNA yielded approximately 10^3 His^+ transformants. A screening step to find the clones producing active dextranase was performed on MD plates containing blue dextran, and the expression was induced with methanol in vapor phase. All the clones tested formed halos of dextran hydrolysis (Fig. 2). In addition, three G418 resistance colonies were obtained on YPD plates containing 4 mg/ml geneticin. This indicates that they contained multiple copies of the gene in the expression vector [20, 24].

Five of these clones (Fig. 2) that showed larger halos were further evaluated in a 100-ml shake-flask experiment. All the clones showed a gradual increase of dextranase activity in the culture supernatant after methanol induction. The clones showed dextranase activity in the range of 1,780 to 2,740 U/l (Table 2).

The PLSD1-2 clone was finally selected for further fermentation studies in an 8-laboratory-scale fermentor based on its higher productivity of extracellular dextranase (2,740 U/l culture).

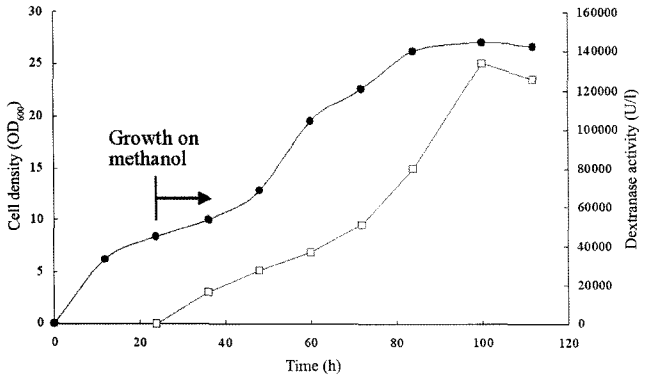

Fig. 3. Growth and dextranase induction in the culture of *P. pastoris* GS115 containing pPIC9K-LSD1.
●, cell density (OD600); □, dextranase activity (U/l).

Expression of Recombinant LSD1

Each colony was evaluated for *lsd1* expression in shaking flasks by methanol induction. Protein expression was initiated by the addition of methanol. We used a fermentor that was equipped with a controller to maintain a given value of dissolved oxygen and temperature. The time course of the fermentation profile for pPIC9K-LSD1 in *P. pastoris* GS115 is shown in Fig. 3. The amount of cells increased rapidly when methanol feeding was started. The entire fermentation process proceeded for 112 h. By methanol induction for 24 h, the dextranase activity in the supernatant reached the highest value of 134,000 U/l (Table 2) at 100 h. The concentration of dextranase in the clarified broth was 0.63 g/l, while the cell density (OD_{600}) was 27.14.

Characterization of Dextranase Produced by *P. pastoris*; A Comparison with the Native Enzyme of *L. starkeyi* and the Dextranase Produced by *S. cerevisiae*

Expressed and native enzymes were recovered from the culture filtrate as they were secreted. Carbohydrate content, as determined by the phenol-sulfuric acid method [5], was estimated to be two or three times higher in the dextranase of the *P. pastoris* transformant; however, the overall glycosylation was not greater than 15%. The open reading frame encoded for a 608-amino-acid polypeptide, with a predicted molecular mass of 67.6 kDa. The recombinant enzyme showed a broad band near 73 kDa. Furthermore, rabbit monoclonal antibodies against a synthetic LSD1 peptide mix also recognized approximately 73 kDa (Fig. 4).

The expression in *S. cerevisiae* of the cDNA copy and genomic copy of the *L. starkeyi* dextranase gene (*lsd1*),

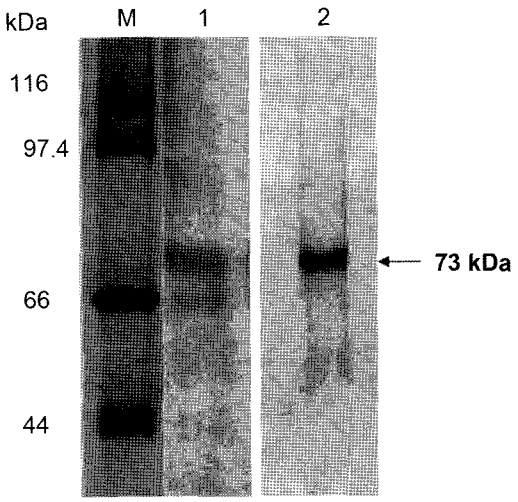

Fig. 4. Electrophoresis separation and immunodetection of LSD1 proteins expressed from the *Pichia* clone.
M, molecular mass markers; Lane 1, Coomassie blue staining of the LSD1; Lane 2, Western blot of a matching gel to show binding of LSD1 protein with antibody against an LSD1 synthetic peptide mix.

linked to the galactose-regulated *GAL*1, resulted in the production of biologically active dextranase [9]. However, the transformation of *S. cerevisiae* containing *lsd1* did not result in dextranase secretion by any of the transformants. When *lsd1* was expressed in *S. cerevisiae* under the control of the pYES2 vector promoter, biologically active dextranase was not secreted into the culture medium, indicating the nonrecognition of the LSD1 signal peptide cleavage in *S. cerevisiae* [9].

The methylotrophic yeast *P. pastoris* has been developed into a highly successful system for the production of a variety of heterologous proteins with high expression levels, either intracellularly or extracellularly. Cloning of the dextranase gene from *Penicillium* and its expression in the *P. pastoris* system was reported previously [7, 21]. In this case, 3.2 g/l of active dextranase was secreted into the medium after induction by methanol. Large-scale expressions and a comparison of the properties of LSD1 from *S. cerevisiae* (32,000 U/l) and that from *P. pastoris* (134,000 U/l) showed different expression levels.

Hydrolysis of Polysaccharides Using the Dextranase of the *P. pastoris* Transformant

The substrate specificity of the dextranase of the *P. pastoris* transformant was determined using different α-glucans formed by α-1,3- α-1,4- and/or α-1,6-glucoside linkages. The enzyme exhibited strong activity for dextran T-2000 as well as 742CB dextran. The relative activity of the dextranase of the *P. pastoris* transformant was low for dextrans having a relatively high content of α-(1→2) or α-(1→4) branching structures, such as 1299CB4 dextran (Table 3). Moreover, the enzyme had very low activity for alternan and pullulan.

Sugar polymers are enormously diverse and widely distributed in different organisms and cell types. In addition, sugar polymers have multiple roles in pathogen detection, immunity, and cell-cell interactions. Dextran belongs to a group of sugar polymers that evolved mainly to benefit a narrow range of microbial species. However, dextran is not unique among these polysaccharides, and most organisms can hydrolyze dextran to a certain extent. Although the structures of dextrans and starch differ considerably, the enzymes hydrolyzing them have many similarities in terms of mechanisms of action and structure. It may, therefore, be necessary to investigate the specific characteristics of a larger number of enzyme groups before common evolutionary linkages can be formulated. Only a few structures of dextranases have been solved [17], and there is practically no information regarding a dextranase such as LSD1, which may possess unique structure-function characteristics.

A few dextranases detected in higher organisms are expected to have novel functions. Purified and specific enzymes having high substrate specificity can be used in basic research for the analysis of more complicated carbohydrate structures as well as in biotechnological applications. The number of applications for dextran and dextran enzymes are expected to increase in the near future. For example, the sugar industry requires more effective thermostable dextranases for various processes. Moreover, processed dextrans may have prebiotic properties and advantageous effects on the texture and consistency of foodstuffs features that the food industry has not yet exploited. Although dextranases have been studied most intensely in the context of dental disease, breakthrough technologies are still waiting to be found. The most interesting dextran-related application may be to create the hydrolysis of microbial dextran, causing microbes to be more prone to antibiotics, which deserves further study.

Acknowledgments

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Table 3. Relative hydrolytic rate of rLSD1.

Substrate (1 mg/ml)	Linkage composition				Hydrolytic rate[a] (μmol/min/mg)	Relative rate[b] (%)
	α-1,6	α-1,3	α-1,2	α-1,4		
Dextran T-2000 from *L. mesenteroides* B-512F	95%	5%			2.68	100
Dextran from *L. mesenteroides* B-742CB	67%	33%			2.36	88.1
Dextran from *L. mesenteroides* B-1299CB4	65%		35%		1.65	61.4
Alternan	50%	50%			0.16	6.1
Pullulan	25%			75%	0.15	5.4

[a]One mg/ml of substrate.
[b]Relative rate calculated when the hydrolytic rate for dextran T-2000 was 100%.

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