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Screening and Identification of a *Streptomyces platensis* YK-2, a New Transglutaminase Producer

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A bacterial strain, YK-2, was isolated as a producer of transglutaminase from a forest soil sample of Daegu, Korea. The isolate showed a G+C content of 72.7 mol%, contained *meso*-A_2pm as the cell-wall amino acid, and possessed menaquinone MK-9 (H_6) and menaquinone MK-9 (H_8) at a ratio of 6:4. The chemotaxonomic analysis, as well as phylogenetic analysis based on the 16S rDNA sequence, identified the isolate as a member of *Streptomyces platensis*. For transglutaminase production, the optimum medium composition was determined to be 2% glucose, 1% polypeptone, 1% soytone, and 0.1% $MnCl_2$. The transglutaminase was stable within the pH range of 5.0–9.0 and 30–45°C, and the optimum pH and temperature were pH 8.0 and 45°C, respectively, without any requirement for Ca^{2+}.

Keywords: Microbial transglutaminase, *Streptomyces platensis* YK-2, 16S rDNA sequence, phylogeny

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase: E.C. 2. 3. 2. 13) catalyzes an acyl-transfer reaction between a γ-carboxyamide group of glutamine (Gln) residues (acyl donors) and a variety of primary amines (acyl acceptors) including an ε-amino group of a Lys residue in certain proteins [4, 8, 10, 12, 24]. This type of enzyme is widely distributed as a superfamily in eukaryotes, and their enzymatic properties have been extensively studied [25]. Incorporation of inter- or intramolecular covalent cross-linkages into food proteins by TGase improves the physical and textural properties of many food products, such as tofu, boiled fish paste, and sausage. The two most extensively investigated TGases are TGase of human epidermis keratinocytes [31], and the human plasma protein Factor XIII [14], which plays important roles in blood clotting. In prokaryotes, TGase activity has been reported only in actinomycetes, such as *Streptoverticillium* sp. [26] and *Streptoverticillum mobaraense* S-8112 [1, 16]. Whereas TGases of mammalian origin are intracellular and Ca^{2+}-dependent, the TGase from *Sv. mobaraense* is extracellular and Ca^{2+}-independent, which stimulated industrial interest in this type of microbial TGase, as these characteristics facilitate as well as decrease the costs of industrial processing. It would thus be worthwhile to develop an efficient production system for microbial TGase. In this study, we screened microorganisms from soil samples obtained in Korea, and we report novel actinomycetes that produce microbial TGase. The cultivation conditions required for microbial TGase production were optimized, and the characteristics of the obtained TGase were investigated.

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Materials and Methods

Screening of TGase-Producing Strains

Strains were isolated from forest soil samples collected at Wayongsan in Daegu, Korea, after growth on a humic acid–vitamin (HV) agar medium [13]. The strains grown on the HV agar plates were transferred and cultured for 5 days at 28°C on plates composed of 0.1% humic acid, 0.05% Na_2HPO_4, 0.17% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.002% $CaCO_3$, 0.005% $MgSO_4 \cdot 7H_2O$, and 2.0% agar (pH 7.2). The TGase-producing strains were detected by a colorimetric hydroxamate assay using *N*-carbobenzoxy-L-glutaminylglycine [9]. Briefly, filter papers dipped into solution A [0.2 M *N*-carbobenzoxy (Cbz)-L-glutaminylglycine (Sigma-Aldrich, Steinhein, Germany), containing 0.1 M calcium chloride, 2.0 M hydroxylamine, and 0.02 M EDTA] were placed on the colonies and incubated at 37°C. After a 2 h incubation period, 20 μl of staining solution [equal portions of 15% (*w/v*) trichloroacetic acid in 0.1 N HCl, and 5% ferrous chloride in

2.5 N HCl] was added to the filter papers. The TGase activity was indicated by the development of a reddish color, which was due to the formation of γ-glutamyl hydroxamate from Cbz-L-glutaminylglycine. The strain YK-2 showed the most intensive red coloring, and was used throughout this study as the producer of extracellular TGase.

Cultivation Conditions

Cultivation was carried out with a basal medium [2% polypeptone, 2% soluble starch, 0.2% yeast extract, 0.2% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 1.5% agar (pH 7.0)]. For the preculture preparation, a spore suspension (10^8 spores) was inoculated into 20 ml of the basal medium in a 100-ml Erlenmyer flask and was cultivated at 28°C for 36 h on a reciprocating shaker (120 strokes/min). The main cultivation was performed with 70 ml of the same medium in a 500-ml Erlenmyer flask by inoculating 2.1 ml of the preculture, followed by incubation at 28°C for 5 days on a rotating shaker at 150 rpm. The culture was filtered by suction filtration (Advance Co., No. 20), and the filtrate was used as a source of crude TGase.

Morphological and Physiological Characteristics

For the morphological characterization, strain YK-2 was cultivated for 14 to 21 days at 28°C on ISP (International *Streptomyces* Project) agar media Nos. 2, 3, 4, 5, and 7 [29]. The morphology of strain YK-2 was examined by light microscopy as well as by scanning electron microscopy (SEM). For the SEM observations, cells from the early growth phase and the stationary phase were prepared by the method described by Bozzola and Russell [3]. The cultural and physiological characteristics were determined at 28°C, as described by Shirling and Gottlieb [29] and Locci [23]. The hydrolysis of starch and tyrosine were tested by the method of Cowan and Steel [6]. The hydrolysis of esculin was determined according to the method of Kurup and Fink [20]. Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1% *p*-aminodimethylaniline oxalate. Nitrate reduction was examined as described by Lanyi [21]. The utilization of various substrates as sole carbon sources was determined as described by Shirling and Gottlieb [29], at a concentration of 1.0% (w/v). Color was determined by comparison with color chips from the ISCC-NBS color charts, standard sample No. 2106 [17].

Chemotaxonomic Characterization

Mycelia for the analyses of menaquinone and fatty acid were obtained after 5 days of growth at 28°C on nutrient agar (Difco). A whole-cell sample was prepared and analyzed as described by Becker *et al.* [2] and Lechevalier and Lechevalier [22]. Isomers of diaminopimelic acid (DAP) in the cell wall peptidoglycan were determined by TLC (Merck; No. 5716) after the hydrolysis of cell wall amino acids with 6 N HCl at 100°C for 18 h [19]. The sugars of the cell wall were analyzed as described by Saddler *et al.* [27]. Menaquinones were analyzed by the procedures described by Watanuki and Aida [34] and Collins [5], using reverse-phase HPLC. Mycolic acids were extracted and purified as their methyl esters according to the procedures of Komagata and Suzuki [19]. Polar lipids and mycolic acids were determined by GC [19] using a gas chromatograph equipped with a flame-ionization detector (Model Varian, Star 3400; U.S.A.). Whole-cell fatty acids were extracted [11], converted to methyl esters by anhydrous methanolic HCl, and analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID).

Isolation of DNA and Determination of the G+C Content

Chromosomal DNA of strain YK-2 was extracted from mycelia grown for 5 days at 28°C in liquid nutrient medium on a reciprocating shaker at 150 rpm, and the DNA was purified according to a previously described method [35]. The concentration and quality of the DNA preparation were determined by spectroscopic measurement and by agarose gel electrophoresis, respectively. After enzymatic hydrolysis and dephosphorylation, the G+C content of the genomic DNA was determined by reverse-phase HPLC according to the method of Tamaoka and Komagata [30] using an equimolar mixture of nucleosides (Yamasa Shoyu Co., Ltd., Chiba, Japan) as the standard.

16S rDNA Sequencing

The sequencing of 16S rDNA was performed as described previously [36], with reverse primer 704R (5'-TCTRCGNATTTCACCNCTAC-3'; positions 704 to 685 in *E. coli* 16S rRNA numbering). In some cases, the sequencing reactions were performed with dITP from a DNA sequencing kit (Amersham), or with the SequiTherm EX-CEL II DNA sequencing kit (Epicentre Technologies), in order to relieve compression artifacts.

Phylogenetic Analysis and Nucleotide Sequence Accession Numbers

The 16S rDNA sequence of strain YK-2 was aligned with the 16S rRNA/16S rDNA sequences of *Streptomyces* species and other related reference strains by using the CLUSTAL W (ver. 1.7) software [32]. The 16S rDNA similarity values were calculated from the alignment. Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. The evolutionary distance matrices were calculated using the algorithm of Jukes and Cantor [15] with the DNADIST program within the PHYLIP package [7]. A phylogenetic tree was constructed using the neighbor-joining method [28] from a distance matrix calculated by CLUSTAL W. The stability of the relationship was assessed by a bootstrap analysis of 1,000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR, and DONSENSE of the PHYLIP package. The reference sequences were obtained from the GenBank, EMBL, and DDBJ databases: *S. platensis* JCM 4662^T (T=type strain, AB045882), *S. lydicus* ATCC 25470^T (Y15507), *S. albulus* ISP 5492^T (AB024440), *S. albofaciences* JCM 4342^T (AB045880), *S. rimosus* JCM 4667^T (AB045883), *S. violaceusniger* ISP 5563^T (AJ391823), *S. hygroscopicus* NRRL 2387^T (AJ391820), *S. melanosporofaciens* NRRL B-12234^T (AJ391837), *S. clavuligerus* JCM 4710^T (AB045869), *S. venezuelae* JCM 4526^T (AB045890), *S. griseocarneus* DSM 40004^T (X99943), *S. sampsonii* ATCC 25495^T (D63871), *S. albidoflavus* DSM 40455^T (Z76676), and *S. somaliensis* DSM 40738^T (AJ007403).

Determination of TGase Activity

The TGase activity was determined by a colorimetric hydroxamate procedure using *N*-carbobenzoxy-L-glutaminylglycine as the substrate [9] with L-glutamic acid-γ-monohydroxamate (Sigma Co.) as the standard. One unit of TGase was defined as the amount of enzyme to form 1 mmole of L-glutamic acid-γ-monohydroxamate per minute at 37°C.

Optimum pH and pH Stability

The pH dependence of the TGase activity was measured using 0.1 M Tris-acetate buffer for the pH range of 4.0-11.0. The enzyme reaction was performed at the indicated pH at 37°C for 10 min. To determine pH stability, the crude TGase was incubated at the indicated pH at 4°C for 10 min, and the solution was adjusted to pH 6.0; then, the remaining activity was measured at 37°C for 10 min at pH 6.0.

Optimum Temperature and Thermostability

To determine the optimum temperature, enzyme reactions were performed at temperatures ranging from 25 to 60°C in 1.0 M Tris–acetate buffer (pH 6.0) for 10 min. Thermostability was determined by incubating the enzyme solution for 10 min, 30 min, and 50 min, at temperatures ranging from 30 to 60°C. After the enzyme solution was rapidly cooled to 4°C, the remaining activity was measured at 37°C for 10 min.

Database Deposition

The 16S rDNA sequence of strain YK-2 was deposited in the DNA Data Bank of Japan (DDBJ Accession No. AB163439).

Results and Discussion

Isolation of Strain YK-2 as a Producer of TGase

In order to obtain a strain that might serve as a new source of TGase, more than 250 strains of actinomycetes were isolated from soil samples obtained in Korea, and were screened for TGase production. Four strains (strains YK-2, YK-57, YK-133, and YK-180) showed TGase activity (data not shown), and the strain YK-2 was selected based on the finding that it exhibited the strongest hydroxamate-forming activity among the strains tested, as determined by plate assay.

Morphological Characteristics of Strain YK-2

Colonies of strain YK-2 are white, wrinkled, and flat on Bennett's agar. Light microscopy of a 7-day culture on ISP 2 agar revealed the presence of the vegetative hyphae. A large number of single spores were borne on vegetative hyphae as well as on the short flexuous aerial mycelium. The substrate mycelium was long, irregularly branched, and did not fragment into short elements. The aerial mycelium branched monopodially and was 0.3–0.4 μm in diameter. Scanning electron microscopy indicated that the spores have smooth surfaces, were oval to cylindrical in shape, and 0.5–0.88 μm in size, with spore chains on the aerial mycelium in tightly closed spirals.

Cultural, Physiological, and Chemotaxonomical Characteristics

Strain YK-2 exhibited good growth on ISP 3 medium, moderate growth on Bennett's, ISP 4, ISP 5, ISP 6, and ISP 7 media, and poor growth on ISP 2 medium (Table 1). This strain did not produce any diffusible pigment on any of the media tested. Strain YK-2 grew optimally at 28–37°C but did not grow at 50°C. In addition, this strain grew optimally at pH 7.2, but not at pH 6.0 or below. The phenotypic and chemotaxonomic properties of strain YK-2 are shown in Table 2. Strain YK-2 was found to have a G+C content of 72.7 mol%, *meso*-diaminopimelic acid as the diamino acid in the cell wall peptidoglycan, and MK-9 (H_6) and MK-9 (H_8) as the major and minor menaquinones in a ratio of 6:4, but lacks mycolic acids. The sugar from the whole cell-wall hydrolysate of strain YK-2 was madurose (3-*O*-methyl-D-galactose), as determined by chromatographic analyses. No diagnostic phospholipid pattern was detected. Strain YK-2 contained *iso*-$C_{14:0}$, *iso*-$C_{15:0}$, *iso*-$C_{16:0}$, and $C_{16:0}$ as the major cellular fatty acids, with small amounts of *iso*-$C_{13:0}$, $C_{14:0}$, *anteiso*-$C_{15:0}$, *iso*-$C_{15:0}$, $C_{15:0}$, *iso*H-$C_{16:1}$, *iso*-$C_{17:1w9c}$, *iso*-$C_{17:0}$, *anteiso*-$C_{17:0}$, $C_{17:0w6c}$, $C_{17:0}$, and *cyclo*-$C_{17:0}$ (Table 3).

Phylogenetic Analysis Based on 16S rDNA Sequences

The 16S rDNA of strain YK-2 was amplified by PCR, and the 1,484 bp of 16S rDNA (corresponding to nucleotides 28 and 1,524 of the *E. coli* 16S rRNA gene) were determined. The unrooted phylogenetic tree (Fig. 1) obtained by the neighbor-joining method [28] indicated that strain YK-2 is similar to type strains of the *Streptomyces* species (similarity: 96.7–99.4%), indicating that strain YK-2 is a member of the genus *Streptomyces* (Table 4). Strain YK-2 was found to form a coherent cluster with the type strain of *S. platensis*, a finding supported by a high bootstrap resampling value of 99.4%. Therefore, strain YK-2 was determined to be *Streptomyces platensis* YK-2, a taxon that is physiologically, chemotaxonomically, and phylogenetically distinct from the genus *Streptoverticillium* and related genera (*Intrasporangium, Kineospora,* and *Sporichthya*).

Cultural Conditions for TGase Production

Considering that microbial TGase production was influenced significantly by the type of carbon sources, nitrogen sources, and mineral salts [18, 37, 38], we investigated the effects of the composition of the medium in order to obtain high levels of TGase production. As shown in Fig. 2, TGase production by strain YK-2 reached a maximum (2.26 units/ml) after 5 days of cultivation, when the growth reached the stationary phase. With the TGase activity

Table 1. Cultural characteristics of strain YK-2.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Bennett's	Moderate	White	Yellow	None
Yeast–malt (ISP 2)	Poor	White	Brown	None
Oatmeal (ISP 3)	Good	White	Yellow	None
Inorganic salts–starch (ISP 4)	Moderate	White	Yellow	None
Glycerol–asparagine (ISP 5)	Moderate	Yellow/White	White	None
Peptone–yeast (ISP 6)	Moderate	White	Pale yellow	None
Tyrosine (ISP 7)	Moderate	White	Abundant yellow	None

Table 2. Morphological, physiological, and chemotaxonomical characteristics of strain YK-2.

Characteristics	YK-2	Characteristics	YK-2
Colony		Inositol	+
Size	1.0–10 mm	Inulin	+
Surface	Wrinkle, Flat	L-Arabinose	+
Spore		L-Rhamnose	±
Size	0.5–0.88 μm	Melibiose	–
Mass color	White, Ivory	Raffinose	+
Surface	Smooth	Salicin	–
Hyphae	Straight	Starch	–
Chain number	4 (spirales)	Sucrose	+
Mycelium		Nitrate reduced to nitrite	+
Aerial	White	Catalase	+
Substrate	White	Oxidase	–
Growth at 37°C	+	Growth in NaCl	
Hydrolysis of		4%	+
Starch	–	10%	+
Tyrosine	+	12%	–
Esculin	–	Diaminopimelic acid	*Meso*
Cellulose	–	Whole-cell sugar pattern	C
Gelatin	–	Cell wall type	III
Carbon utilization		Phospholipid type	PII
Adonitol	–	Menaquinone	
Cellobiose	–	Major	MK9 (H_6)
D-Galactose	–	Minor	MK9 (H_8)
D-Glucose	+	G+C content	72.7 mol%
D-Fructose	+		
D-Mannitol	±		

All results are from study.
+, Positive reaction; –, negative reaction; ±, weekly positive reaction.

attained after 5 days of cultivation, glucose at the initial concentration of 20 g/l gave the highest levels of production, whereas lactose gave the lowest levels of production (Table 5). These results differed from those observed when the best carbon source for production was either soluble starch [1, 37] or glycerol [33].

Only organic nitrogen sources such as soytone, beef extract, polypeptone, and yeast extract produced a high yield of TGase, and inorganic sources produced only poor yields (Table 6). By surveying several combinations of these nitrogen sources, a combination of polypeptone and soytone (each 10 g/l) was found to give the highest levels of TGase production. By decreasing the total nitrogen concentration but simultaneously keeping the ratio between polypeptone and soytone constant, 10 g/l of total nitrogen sources (polypeptone 5 g/l and soytone 5 g/l) were determined

Table 3. Cellular fatty acid methyl esters of strain YK-2.

Fatty acids	RT (min)	Composition (%)	Fatty acids	R.T. (min)	Composition (%)
12:0 *Iso*	4.137	0.16	16:0	10.070	10.81
13:0 *Iso*	5.165	0.59	17:1 *Iso* w9c	10.781	0.54
13:0 *Anteiso*	5.262	0.07	17:1 *Anteiso* w9c	10.957	0.29
13:0	5.596	0.10	17:0 *Iso*	11.146	5.97
14:0 *Iso*	6.418	14.09	17:0 *Anteiso*	11.304	1.93
14:0	6.925	0.87	17:1 w8c	11.422	0.14
15:0 *Iso*	7.866	16.40	17:1 w6c	11.540	0.57
15:0 *Anteiso*	8.001	6.62	17:0 Cyclo	11.584	0.58
15:0	8.434	2.39	17:0	11.778	1.15
16:1 *Iso H*	9.184	0.46	18:0 *Iso*	12.876	0.21
16:0 *Iso*	9.462	33.61	18:0	13.516	0.29

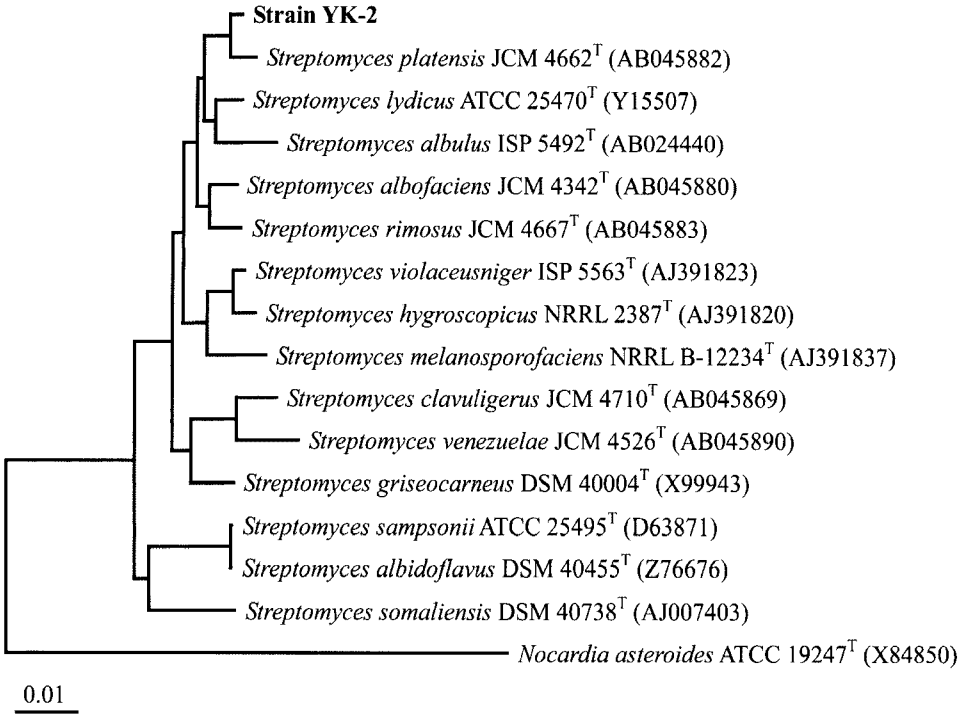

Fig. 1. Phylogenetic tree showing the relationships between strain YK-2 and related species.

to be optimal for TGase production. In addition, the production of microbial TGase has been reported to be influenced by the presence of minerals and trace elements such as phosphate, magnesium, potassium, iron, copper, zinc, and vitamins [1, 26]. In the case of *S. platensis* strain YK-2, the production of TGase was low in the presence of iron and zinc, whereas high levels of TGase were produced in the presence of manganese at an optimum concentration of 0.1% (*w/v*) (Table 7). Although the relationship between Mn^{2+} and TGase synthesis is not clear at present, Mn^{2+} has been suggested to help stabilize the TGase produced in the cell broth.

Based on the above results, the optimum medium for obtaining TGase production by strain YK-2 was determined to be 2% glucose, 1% polypeptone, and 1% soytone, and 0.1% $MnCl_2$. With this medium, strain YK-2 exhibited high enzyme activity (TGase of 4.0–5.0 units/ml); that is

Table 4. Percentage 16S rDNA/16S rRNA similarity between strain YK-2 and reference strains used in the phylogenetic analysis.

Species (Taxon)	(%) Sequence similarity														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Strain YK-2															
2. *Streptomyces platensis* JCM 4662T	99.4														
3. *Streptomyces lydicus* ATCC 25470T	99.1	98.7													
4. *Streptomyces albulus* ISP 5492T	98.0	97.8	98.6												
5. *Streptomyces albofaciens* JCM 4342T	98.4	98.0	98.9	98.3											
6. *Streptomyces rimosus* JCM 4667T	98.6	98.6	98.2	98.0	99.1										
7. *Streptomyces violaceusniger* ISP 5563T	98.4	98.1	97.7	97.3	98.2	98.4									
8. *Streptomyces hygroscopicus* NRRL 2387T	98.1	98.2	97.4	97.1	97.7	98.4	99.5								
9. *Streptomyces melanosporofaciens* B-12234T	97.6	97.6	97.7	97.4	97.6	98.2	98.3	98.4							
10. *Streptomyces clavuligerus* JCM 4710T	97.4	97.2	97.5	97.6	96.8	97.1	97.0	97.0	96.9						
11. *Streptomyces venezuelae* JCM 4526T	97.2	97.0	96.8	97.0	96.4	96.9	96.6	96.6	96.4	98.4					
12. *Streptomyces griseocarneus* DSM 40004T	98.6	98.4	97.8	97.7	97.4	98.1	98.0	97.8	97.4	97.8	97.8				
13. *Streptomyces sampsonii* ATCC 25495T	96.9	97.4	96.7	96.6	96.8	97.2	97.2	97.6	96.8	96.0	96.1	96.7			
14. *Streptomyces albidoflavus* DSM 40455T	96.9	97.4	96.7	96.6	96.9	97.2	97.3	97.6	96.8	96.0	96.1	96.6	100		
15. *Streptomyces somaliensis* DSM 40738T	96.7	96.7	96.2	96.5	96.7	97.1	96.9	97.2	96.7	96.9	96.5	97.1	97.4	97.4	
16. *Nocardia asteroides* ATCC 19247T	89.6	89.5	89.0	89.4	90.0	90.3	89.3	89.6	88.9	89.3	89.1	89.7	89.8	89.7	90.1

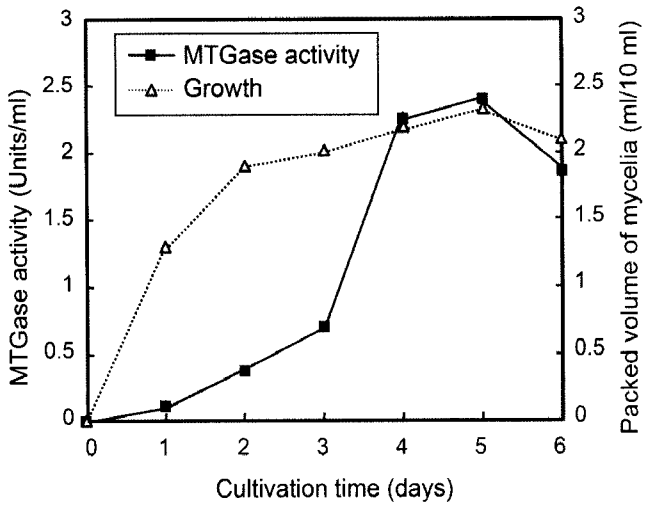

Fig. 2. Time course of growth and TGase production during the cultivation of strain YK-2.
Cultivation was carried out at 28°C in a basal medium that consisted of 2% polypeptone, 2% soluble starch, 0.2% yeast extract, 0.2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

levels significantly higher than those produced by other microorganisms (0.28–2.5 units/ml) [38].

Optimal pH and Temperature, and Thermal and pH Stabilities

The optimum pH of the TGase at 37°C was determined to be pH 8.0 (Fig. 3A). The enzyme was stable at pH 5.0–9.0 after a 10 min incubation at 4°C, at which more than 80% of the activity was retained (Fig. 3B). The optimum temperature of the TGase was determined to be 45°C (Fig. 3C). The enzyme was stable below 45°C and lost 40% and 90% of its activity when incubated at 50°C and 60°C for 10 min, respectively (Fig. 3D). The optimum pH of 8.0 and temperature of 45°C of this enzyme differ from those of *Streptoverticillium* sp. (pH 6–7 and 50°C) [33], although the pH stability of the present strain (5.0–9.0) was similar to that of *Streptoverticillium* sp. [1]. The thermal stability

Table 5. Effect of carbon sources on the production of TGase.

Carbon source	TGase activity (units/ml)			
	Cultivation time (days)			
	2	3	4	5
Dextrin (20 g/l)	0.68	0.58	0.49	0.21
Galactose (20 g/l)	0.29	0.81	0.49	0.86
Glycerol (20 g/l)	0.68	1.73	2.10	1.63
Lactose (20 g/l)	0.50	0.25	0.07	0.06
Na-acetate (20 g/l)	0.06	0.07	0.07	0.07
Soluble starch (20 g/l)	0.49	0.80	0.58	2.51
Sucrose (20 g/l)	0.62	0.62	0.60	0.56
Glucose (10 g/l)	0.99	1.54	2.80	2.25
Glucose (20 g/l)	0.96	1.51	4.38	5.05
Glucose (30 g/l)	1.12	1.88	2.44	3.27
Glucose (40 g/l)	1.04	1.68	2.28	1.41
Glucose (50 g/l)	0.41	0.68	1.39	0.99

Table 6. Effect of nitrogen sources on the production of TGase.

Nitrogen source	TGase activity (units/ml)	
	Cultivation time (days)	
	3	5
Inorganic		
NH_4Cl (20 g/l)	0.14	0.06
$(NH_4)_2SO_4$ (20 g/l)	0.09	0.12
$NaNO_3$ (20 g/l)	0.09	0.09
Organic		
Beef extract (20 g/l)	2.67	4.37
Casamino acid (20 g/l)	0.28	1.40
Casitone (20 g/l)	0.76	1.76
Malt extract (20 g/l)	0.17	1.33
Polypeptone (20 g/l)	2.51	4.76
Soytone (20 g/l)	2.79	4.48
Urea (20 g/l)	0.09	1.14
Yeast extract (20 g/l)	1.54	4.57
Polypeptone (10 g/l)+Beef extract (10 g/l)	1.43	3.33
Polypeptone (10 g/l)+Yeast extract(10 g/l)	2.45	3.50
Beef extract (10 g/l)+Soytone (10 g/l)	1.49	3.76
Yeast extract (10 g/l)+Soytone (10 g/l)	1.28	3.89
Yeast extract (10 g/l)+Beef extract(10 g/l)	1.52	4.32
Polypeptone (10 g/l)+Soytone (10 g/l)	2.20	4.63
Polypeptone (2.5 g/l)+Soytone (2.5 g/l)	1.80	3.80
Polypeptone (5 g/l)+Soytone (5 g/l)	2.30	4.84
Polypeptone (15 g/l)+Soytone (15 g/l)	1.95	4.09
Polypeptone (20 g/l)+Soytone (20 g/l)	0.68	1.43
Polypeptone (25 g/l)+Soytone (25 g/l)	0.83	1.76

Table 7. Effects of inorganic salts on the production of TGase.

Inorganic salts	TGase activity (units/ml)		
	Cultivation time (days)		
	3	4	5
None	0.58	4.00	4.22
$CaCl_2$ (1 g/l)	0.56	2.38	3.34
$FeCl_3$ (1 g/l)	0.83	1.08	0.80
$FeSO_4$ (1 g/l)	0.57	0.81	0.86
$MgSO_4$ (1 g/l)	1.40	4.48	4.19
$ZnSO_4$ (1 g/l)	0.56	0.91	1.28
$MnCl_2$ (1 g/l)	1.57	4.44	4.96
$MnCl_2$ (2 g/l)	1.34	3.79	3.81
$MnCl_2$ (3 g/l)	1.25	3.55	3.17
$MnCl_2$ (4 g/l)	0.42	1.23	2.13
$MnCl_2$ (5 g/l)	0.06	0.17	0.32

Cultivation was done at 28°C in a medium containing 2% glucose, 0.5% polypeptone, and 0.5% bacto-soytone at pH 7.0.

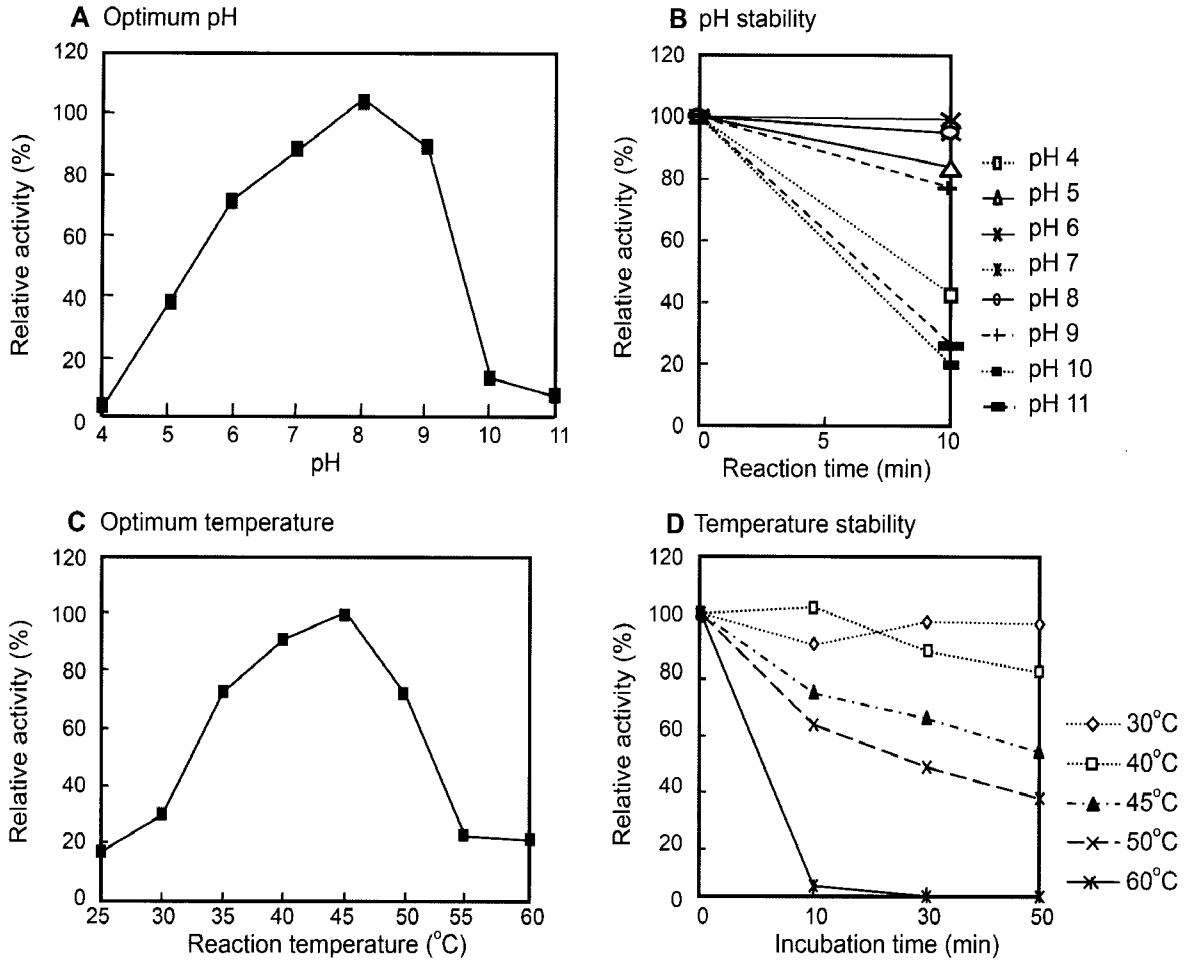

Fig. 3. Characterization of the transglutaminase produced by strain YK-2.
A. Effect of pH on TGase activity. TGase activity was measured at the indicated pH for 10 min at 37°C. The buffer used was 0.1 M Tris–acetate (pH 4.0–11.0). **B**. pH stability of TGase. After incubation at the indicated pH for 10 min at 4°C, the pH was adjusted to pH 6 and the remaining TGase activity was measured at 37°C. **C**. Effect of temperature on TGase activity. TGase activity was measured at various temperatures (25–60°C) for 10 min in a 1.0 M Tris–acetate buffer (pH 6.0). **D**. Temperature stability of TGase. TGase was incubated at various temperatures for 10 min, 30 min, and 50 min in a 1.0 M Tris–acetate buffer (pH 6.0), and the remaining activity was measured at 37°C.

(60% activity at 50°C for 10 min) was lower than that of *Streptoverticillium* sp. (74% activity at 50°C). From these results, it can be concluded that the TGase of strain YK-2 differs from other known TGases, such as that from *Streptoverticillium* sp.

Influence of Calcium Ions

One important feature of microbial TGase is that it is Ca^{2+} independent [1], whereas TGases from animals require Ca^{2+} as a cofactor for the transfer reaction [24]. Therefore, the influence of calcium ions on the TGase of strain YK-2 was investigated. The enzymatic activity of this strain was found to be similar in both the presence and the absence of calcium ions, and the activity was not inhibited by EDTA (data not shown). Thus, the present results revealed that this enzyme should be defined as a Ca^{2+}-independent enzyme, similar to the TGase of *Streptoverticillium* sp. The results obtained here may help identify a novel regulatory substance of TGase in biological systems.

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