Mutation of AN39-1 for production and characterization of constitutive, thermostable and pH-resistant dextranases

Abstract: Objective: Leuconostoc mesenteroides AN39-1 has recently been isolated from Crataegus orientalis var. Orientalis. It produces inducible extracellular dextranase (EC 2.4.1.5) forming dextran from sucrose. The aim of this study was (1) to obtain constitutive, pH-resistant and thermostable dextranase, (2) to characterization of these dextranases.

Methods: Mutagenesis was carried out on the parent strain (AN39-1) using UV, ethyl methane sulfonate, and N-methyl-N’-nitro-N-nitrosoguanidine. Dextranases from wild type (AN39-1) and the mutant strain (A26-2/11) were purified by polyethylene glycol (PEG) precipitation and characterized.

Results: Mutants (A26, A26-2, and A26-2/11) hyper producing and constitutive for dextranase were isolated. The mutants (A26, A26-2, A26-2/11) produced 7.2, 8.1, and 2.0 times more dextranase activity as compared to parent strain on sucrose medium, respectively. In addition, the mutants produced dextranase on glucose medium with higher activities (3.0-5.8 times) than what the parental strain produced on sucrose medium. The mutant enzyme (A26-2/11) was much more thermostable than the native enzyme and resistant to pH more than dextranase of AN39-1. The dextranase from mutant strain was stable up to 35°C and pH of 7.5 for 3 hr.

Conclusion: The structures of dextrans produced by wild type and mutant enzymes were similar to commercially produced B-512 F dextran. Thus, the newly dextranases produced by mutant strain could find industrial applications at higher temperature and pH.

Keywords: Leuconostoc mesenteroides, dextranase, mutation, ethyl methane sulfonate, N-methyl-N’-nitro-N-nitrosoguanidine, ultraviolet
Introduction

Dextran is a polymer composed of glucose units connected by α (1→6) linkages with varying amounts and arrangements of α (1→2), α (1→3), or α (1→4) branch linkages [1]. They are produced from sucrose by dextransucrases (EC 2.4.1.5) which are produced by various species of Leuconostoc, Streptococcus, and Lactobacillus. The dextransucrases from Streptococcus are produced constitutively. However, dextransucrases from Leuconostoc mesenteroides (L. mesenteroides) can only be produced by sucrose induction. Strains of L. mesenteroides that must be induced by sucrose in the culture medium have at least three major problems: Only one half of the carbon source, the fructose part of sucrose, can be utilized for growth. The culture fluid becomes highly viscous during growth due to an increase in the amount of dextran. The enzyme produced has dextran attached due to the presence of dextran produced from sucrose which is substrate for dextran production and an inducer for dextransucrase production. Thus, enzyme production from Leuconostoc mesenteroides is accompanied with dextran production to which the enzyme has a high affinity. The enzyme is difficult to purify because of the high viscosity and presence of a large amount of dextran [2]. In order to overcome the problems associated with dextran, several mutation techniques have been used to produce constitutive dextransucrases.

Kim and Robyt [1] obtained several L. mesenteroides (B-512FM, B1142, B-1355 and B-742) constitutive mutants by using ethyl methansulfonate (EMS). Iliev et al. [3] mutated L. mesenteroides strain Lm 28 and Bl-08 with EMS to obtain constitutive mutant forms. L. mesenteroides B-1355 was also mutated using N- methyl-N´-nitro-N-nitrosooguanidine (NTG) by Smith and Zahnley [4].

Leuconostoc mesenteroides (Lm) NRRL B-512F dextransucrase is the only enzyme used commercially to produce dextran [5]. B-512F dextran has 95% α (1→6) linkages and 5% α (1→3) branch linkages. It has a number of industrial uses such as production of sephadex and blood plasma substitute [6,7].

Recently, our research group has isolated a strain from Crataegus orientalis var. Orientalis, (AN39-1: Leuconostoc mesenteroides ssp. mesenteroides; GenBank accession no: GQ280007) producing dextransucrase which catalyzes the formation of dextran from sucrose [8]. The enzyme is produced extracellularly and is inducible by sucrose. The structure of the dextran produced by this enzyme is similar to commercial dextran produced by B-512F dextransucrase. It was also found that the newly produced AN39-1 dextransucrase could carry out dextran synthesis at low temperatures (4°C) better than Lm B-512 F dextransucrase.

The purpose of this study was to find mutant strains of AN 39-1 constitutive for dextransucrase to overcome problems associated with the production of inducible dextransucrase. The present work describes mutagenesis of L. mesenteroides AN39-1 using UV, EMS, and NTG to isolate hyperproducing strains constitutive for dextransucrase. Dextransucrase produced by wild type (AN39-1) and the mutant strain (A26-2/11) were purified and characterized.

Materials and Methods

Bacterial strains

Strain AN39-1 was isolated from a native plant (Crataegus orientalis var. Orientalis) [8]. The mutant, A26, was obtained from strain AN39-1 by UV mutagenesis. The mutant, A26-2, was obtained from A26 by mutagenesis with EMS. The mutant, A26-2/11, was isolated in mutagenesis of A26-2 using NTG.
**Chemicals and enzyme**

Glucose, ammonium peroxodisulfate, glycine, Coomassie brilliant blue, dinitrosalycilic (DNS), and α-naphthol were obtained from Fluka (Buchs SG, Switzerland). Calcium acetate, sucrose, sulfuric acid, ethanol, acetonitrile, acrylamide, bromophenole blue, magnesium sulfate heptahydrate, ferrous sulphate heptahydrate, sodium chloride, manganese sulfate, dipotassium hydrogen phosphate, PEG and calcium chloride were purchased from Merck (Frankfurter Str, Darmstadt, Germany). N-N-N-N-tetramethylendiamine, dextran (Mw: 100,000-200,000), sodium dodecyl sulfate (SDS), methanol, dialysis tubing and molecular weight markers (Mw: 29,000-200,000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetracycline and streptomycin were obtained from Oxoid (Basingstoke, Hampshire, England). Eupergit C was a gift from Roehm GmbH & Co., KG (Darmstadt, Germany). Thin layer chromatography (TLC) plates (Whatman K5) were purchased from Whatman (Clifton, NJ, USA). Ethyl methane sulfonate (EMS) and protein reagent for the Bradford dye-binding assay were from Sigma Chemical Company, St. Louis, Mo, Bio-Rad (Richmond, Calif.). N-methyl-N’-nitro-N-nitrosoguanidine (NTG) was obtained from ABCR (Karlsruhe – Germany).

Dextranase 50 L, a commercial enzyme produced by submerged fermentation of a selected strain of *Penicillium lilacinum*, was a gift of Novozyne (Bagsvaerd, Denmark). The activity of the enzyme was 63 IU/ml. The activity unit (IU) is defined as the amount of enzyme forming 1 μmol isomaltooligosaccharide (isomaltose and isomaltotriose) from dextran solution [Mw: 5,000, 2%(w/v)] in 1 min at pH 5.4 and 30°C.

**Mutagenesis**

**Mutagenesis using UV**

UV mutagenesis on AN39-1 was carried out on cultures grown overnight at 30°C in MRS broth (100 ml) as described by Smith et al. [4]. The cells were harvested aseptically by centrifugation at 4,000 x g for 20 min and then washed three times using cold (4°C), sterile NaCl solution [150 ml, 0.85% (w/v)]. The final pellets were resuspended in sterile NaCl solution (100 ml) and diluted to a concentration of approximately 10^6 CFU/ml. The suspensions (10 ml) were placed into sterile glass petri dishes (150 mm in diameter) and irradiated by 254-nm UV light using Mini UV/Vis® ultraviolet light lamp (Desaga GmbH, Heidelberg, Germany) for different time intervals at varied distances ranging from 20 to 360 sec and 7 to 40 cm. The radiation dose was chosen to give rise to a 0.1% surviving rate (distance: 40 cm; time: 160 sec) as estimated from a UV killing curve. Irradiation and subsequent steps were carried out in darkness to prevent photoreactivation. After irradiation, the cell suspensions (1 ml) were transferred to a MRS broth (4 ml) and then incubated in darkness at 30°C for 48 h. Cell suspensions were then diluted serially (10-fold dilutions) into sterile MRS broth and the dilutions were plated onto MRS agar containing sucrose [10% (w/v)] and maltose [10% (w/v)]. The plates were incubated in darkness at 30°C for 48 h. Approximately 200 colonies were screened and 105 colonies were isolated as mutants based on differences such as size and appearances. Unirradiated control cultures were also studied at the same time as the irradiated cultures to serve as a standard for comparing colony morphologies.

**Mutagenesis using ethyl methane sulfonate**

Mutagenesis with ethyl methane sulfonate (EMS) on the mutant strain, A26, was carried out as described by Kim & Robyt, [1]. The mutant A26, a UV variant of AN39-1, was grown on glucose medium and then collected from 1-day-old liquid culture by centrifugation at 10,000 rpm for 10 min. The cell suspension (1.5 ml), washed twice with sterile sodium phosphate buffer solution (1.5 ml, 100 mM, pH 7.0), was treated with EMS solution (40 µl, 1.17 gr EMS/ml) at 28°C for 1 h. The treated cell suspension (1 ml) was then transferred to a sterile sodium thiosulfate solution [3 ml, 10% (w/v)] to neutralize the remaining EMS. The cells, then were centrifuged (10,400 g), washed twice with sodium phosphate buffer solution (100 mM, pH 7.0) and plated onto glucose and sucrose agar. The plates were

**Culture media**

MRS broth and MRS agar were obtained from Fluka Laboratories. Sucrose-agar (1 L) was prepared using sucrose (50 g), yeast extract (4.2 g), peptone (4.2 g), MgSO₄·7H₂O (0.17 g), FeSO₄·7H₂O (0.008 g), NaCl (0.008 g), MnSO₄·H₂O (0.008 g), CaCl₂·2H₂O (0.011 g), K₂HPO₄ (16.7 g), agar (15 g), and distilled water. Glucose medium (1 L) consisted of glucose (18.8 g), yeast extract (4.2 g), peptone (4.2 g), MgSO₄·7H₂O (0.17 g), FeSO₄·7H₂O (0.008 g), NaCl (0.008 g), MnSO₄·H₂O (0.008 g), CaCl₂·2H₂O (0.011 g), K₂HPO₄ (16.7 g), and distilled water [9]. Sucrose medium (1 L) is composed of sucrose (25 g), yeast extract (4.2 g), peptone (4.2 g), MgSO₄·7H₂O (0.17 g), FeSO₄·7H₂O (0.008 g), NaCl (0.008 g), MnSO₄·H₂O (0.008 g), CaCl₂·2H₂O (0.011 g), K₂HPO₄ (16.7 g), and distilled water [9].
incubated at 30°C for 48 h. Colonies on the plates were examined on the basis of morphology. Slimy shiny colonies on sucrose agar were then picked and single colony was restreaked on each agar plates for isolation of dextran producing Leuconostoc strains. 46 colonies having dextransucrase activities were isolated.

**Mutagenesis using N-methyl-N’-nitro-N-nitrosoguanidine**

A26-2, an EMS variant of A26, was mutated with N-methyl-N’-nitro-N-nitrosoguanidine (NTG) as described by Kitaoka & Robyt [2]. A26-2 was cultivated in the glucose medium (1.2 ml) at 30°C for 24 h and a portion of the culture (200 µl) was centrifuged (10,400 g) and washed three times with a sterile sodium-citrate buffer solution (200 µl, 20 mM, pH 6.0). The cells, then were treated with NTG solution (200 µl, 500 µg NTG/ml) at 21°C for 30 min. The mutated cells were centrifuged at 8000 rpm for 8 min and washed three times with sterile sodium-citrate buffer (200 µl, 20 mM, pH 6.0) followed by cultivation in the glucose medium (500 µl) at 21°C for 3 h. The culture was diluted (1:105) and portions (100 µl) were plated onto glucose medium containing agar [2.5% (w/v)]. 24 colonies having dextransucrase activities were isolated. The enzyme activity was determined using the method described in 2.5

**Selection of mutants constitutive for dextransucrase production**

Constitutive mutants were selected using the agar overlay method of Mizutani et al. [10]. Serial dilutions 10-fold in NaCl solution [(0.85%, (w/v)] of mutagenized cell suspensions were plated onto MRS agar plates (Difco Laboratories, Detroit, Mich.), incubated at 30°C for 48 h, then overlaid with soft agar [1%, (w/v)] containing sucrose [10%, (w/v)], tetracycline [0.4% (w/v)], and streptomycin sulfate [0.2%, (w/v)]. After incubation at 22°C for 6 h, some colonies formed polysaccharide zones, indicating dextransucrase activities were isolated. Poly saccharide zones were detected visually.

**Determination of dextransucrase activity**

The assay mixture (0.5 ml) contained enzyme solution (50 µl) and sucrose (150 mM) in calcium acetate buffer (25 mM, pH 5.4). The reaction mixture was incubated at 30°C for 20 min. Aliquots (0.1 ml) from the reaction mixture were analyzed for reducing sugar by DNS method [11]. The activity unit (IU) is defined as the amount of enzyme catalyzing the formation of 1 μmol of D-fructose from sucrose (150 mM) in acetate buffer (25 mM, pH 5.4) at 30°C per min.

**Protein concentration determination**

The protein concentration was assayed by the method of Bradford using bovine serum albumin (Sigma) as the standard [12]. Absorbance at 595 was measured after 5 min of color development.

**Zymographic analysis**

Non-denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a resolving gel [1.5 mm thick, 7.5%(w/v) acrylamide] at room temperature (25°C) using a vertical slab mini gel unit (BioRad), according to the method of Laemmli [13]. Proteins were stained using Coomassie brilliant blue G 250. In situ detection of dextransucrase activity in the gel was carried out using the protocol described by Purama & Goyal [9]: The gel was washed with acetate buffer containing CaCl₂ (0.3 mM) and Tween 80 [0.1%(w/v)] [100 ml, 25 mM, pH 5.4] at 4°C for 30 min to remove SDS. Then, it was incubated in acetate buffer (100 ml, 25 mM, pH 5.4) containing sucrose [10% (w/v)] at 30°C for 44 h to form dextran within the gel on locations having dextransucrase activity. The sucrose-treated gel was washed first with a solvent system of methanol: acetic acid: water [200 ml, 50:10:40 (v/v/v)] for 30 min, then with water (100 ml) for 30 min. Finally, the gel was treated with periodic acid solution [20 ml (0.5% (w/v) containing acetic acid [1.5% (w/v)] at 30°C for 45 min and washed with water (200 ml in total) 5 times. The cleaned gel was stained with Schiff’s reagent (10 ml) consisting of Fuschin basic [1% (w/v)], sodium bisulphite [2% (w/v)], and HCl (0.13 M) for 5 min to form magenta bands within the gel matrix, an indication for dextransucrase activity.

**Carbohydrate analysis**

Thin layer Chromatography (TLC) was used for analysis of dextran hydrolysis products [14]. TLC plates were developed using 3 ascents of nitromethane-water-t-propanol [2:3:5 (v/v/v) up to two thirds of the plate, and then 3 ascents of acetonitrile/water [85:15 (v/v)] to the top of the plate. Carbohydrates on TLC plates were visualized by dipping the plates into sulphuric acid [5% (v/v)] in ethanol containing α-naphthol [0.5% (w/v)], followed by heating on a
hot plate at 110°C for 10 min. TLC-imaging densitometer, Bio-Rad GS-670, (Bio-Rad, Hercules, CA, USA) was used for quantitative determination of carbohydrates.

**Purification of dextranucrases**

Dextranucrases (AN39-1 and A26-2/11) produced in sucrose medium were purified by PEG fractionation. The cell-free extracts (200 ml) were added ice cold polyethylene glycol solutions (PEG-400 and PEG-6000) so as to obtain cell free extract having PEG [5 and 25% (w/v)] and incubated for 16 h at 4°C to allow the dextranucrase fraction to precipitate. The mixture was centrifuged at 13,200 g at 4°C for 30 min to separate the dextranucrase fraction. The pellets were dissolved in calcium acetate buffer (25 mM, pH 5.4). These fractions were analyzed for dextranucrase activity and protein content and subjected to dialysis using 12 kDa cutoff membrane. The dextranucrase fractions were analyzed for activity and protein content.

**Effects of temperature and pH on dextranucrase activities**

Effects of temperature and pH on dextranucrase activities were determined by changing individually the conditions of the dextranucrase activity assay (pH from 3.0 to 8.0; temperature from 20 to 50°C). The stabilities of the enzymes were established by measuring the residual activities at optimum pH (pH 5.5 for AN 39-1 and 4.0 for A26-2/11) and temperature (35°C) after incubating the enzymes at different pH (3.0–8.0) for 3 h and temperatures (20–50°C) for 40 min.

**Results and Discussion**

AN39-1 (*Leuconostoc mesenteroides* ssp. *mesenteroides*), recently isolated by our group from *Crateagus orientalis* var. *Orientalis*, produces dextranucrase catalyzing the formation of dextran from sucrose [8]. The enzyme is produced extracellularly and is inducible by sucrose [8]. The structure of the dextran produced by this enzyme is similar to commercial dextran produced by B-512F *Leuconostoc mesenteroides* dextranucrase.

In this research, mutagenesis using various mutagens on AN39-1 was carried out to obtain mutants constitutive for dextranucrase. Cell suspensions of AN39-1 were exposed to UV light from 20 sec to 6 min and found that irradiation time of 160 sec gave 0.1% survivors from which 105 colonies were selected at the basis of colony morphology (size and opacity). The enzyme activities were determined in the cell free culture broth. It was found that 2 out of 105 colonies were constitutive for dextranucrase as indicated by agar overlay method. The constitutive mutant strains, called A6 and A26, showed 32% and 62% increase in dextranucrase production, respectively. In mutagenesis of A26 using EMS, 46 colonies were isolated and A26-2 having 71% increased dextranucrase activity was selected for further mutagenesis. Treatment of A26-2 with NTG resulted in new mutant strains. 24 colonies were chosen and A26-2/11 was further studied Table 1. shows dextranucrase activities produced by mutant bacteria grown in sucrose and glucose mediums. Grown on sucrose, all the mutants produced more dextranucrase than the parent strain, AN39-1. The activities of mutant enzymes (A26 and A26-2) were higher (7.2 and 8.1 times) when grown on sucrose medium than that of parent strain. A26 and A26-2 grown on sucrose had twice more enzyme activities than those grown on glucose. Mutagenesis studies on dextranucrases were also carried out by other researchers. Kamal et al. [15] mutated *Leuconostoc mesenteroides* PTCC 1059 with UV light and found that dextranucrase activities of mutant strains showed 20% to 40% increase [15]. Iliev et al. [3] reported that treatment of *L. mesenteroides* strain Lm 28 with EMS resulted in mutant strains producing 40% more dextranucrase than the parent strain [3]. Mizutani et al. [10] obtained constitutive mutants for dextranucrase from *Leuconostoc mesenteroides* NRRL-B-512F using NTG. Dextranucrase activity of one mutant strain was 3-fold higher than that of the wild strain [10]. Kim & Robyt [1] studied on the production and selection of mutants of *Leuconostoc mesenteroides* constitutive for glucansucrases. They isolated mutants constitutive for glucansucrases from *Leuconostoc mesenteroides* NRRL B-512FM, B-1142, and B-1355. The mutants produced higher glucansucrase activities (3 to 22 times) when grown on glucose than the parent strains grown on sucrose [1].

**Table 1: Dextranucrase activity of AN 39-1 and its constitutive mutants.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutagen</th>
<th>Activity (IU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN39-1</td>
<td>None</td>
<td>1.60</td>
</tr>
<tr>
<td>A26</td>
<td>UV</td>
<td>11.53</td>
</tr>
<tr>
<td>A26-2</td>
<td>UV+EMS</td>
<td>12.98</td>
</tr>
<tr>
<td>A26-2/11</td>
<td>UV+EMS+NTG</td>
<td>3.25</td>
</tr>
</tbody>
</table>

*aThe strains were grown in static condition at 30°C for 16 h.*
Analysis of zymogram

Zymogram was used for *in situ* detection of dextranase activity in SDS-PAGE gel. The gel was treated first with sucrose and then periodic acid-Schiff’s base reagents, resulting in staining in purple in color. The formation of purple bands on the gel shows the presence of dextran synthesis by active dextranases from sucrose within PAGE (Fig. 1a). The molecular sizes of dextranases were determined using activity staining gel and non-denaturing SDS-PAGE containing protein standards, stained with Coomassie brilliant blue (Fig. 1b). It was found that AN39-1 had 1 major (184 kDa) and 1 minor active band (180 kDa). Its mutants (A26, A26-2, and A26-2/11) had also 1 major (184 kDa) and 1 minor (150 kDa) active bands. *In situ* detection of enzyme activity for the characterization of dextran-producing *Leuconostoc* strains has been used in some studies [16,17]. Activity staining gel (Fig. 1a) and non-denaturing SDS-PAGE (Fig. 1b) show that isolated mutants produce much more dextranase than AN39-1.

Enzyme purification and characterization

The purification of crude enzymes from AN39-1 and A26-2/11S by polyethylene glycol fractionation resulted in significant increase in dextranase activities. The results of purification process are presented in Table 2. The fractions of AN39-1 and A26-2/11S with 25% (V/V) PEG 400 gave maximum specific activities of 29.77 IU/mg and 27.00 IU/mg resulting in 50 and 29-fold purifications, respectively. The fractions of AN39-1 and A26-2/11S with 5% (V/V) PEG 6000 gave the maximum specific activities of 5.85 IU/mg and 14.54 IU/mg, resulting in 10 and 16-fold purifications, respectively. The characterization studies were carried out using enzymes purified using 25% (V/V) PEG-400 (Fig. 2).

The optimum temperature for both of the enzymes was 35°C (Fig. 2a). The enzyme activities gradually decreased after 35°C. This result is supported by the findings that optimum temperature for dextranase activity from *Leuconostoc mesenteroides* B-512F is in the range of 30–35°C [5]. The mutant enzyme (A26-2/11) was much more thermostable than the native enzyme, AN39-1 (Fig. 2b). At 50°C, AN39-1 lost all of its initial activity. However, A26-2/11S lost about 90% of its initial activity at the same temperature.

The optimum pH for AN 39-1 and A26-2/11 dextranases were 5.5 and 4.0, respectively (Fig. 2c). The mutant enzyme, A26-2/11, was resistant to pH more than dextranase of AN 39-1 (Fig. 2d). AN 39-1 and A26-2/11 dextranases were stable in the pH range of (4.0–7.0) and (4.0–7.5), respectively. The optimum pH’s of the enzymes studied are quite different from that of the commercial enzyme, *Leuconostoc mesenteroides* NRRL B-512F dext-

<table>
<thead>
<tr>
<th>PEG</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Top. Activity (IU)</th>
<th>Sp. Act (IU/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude (AN39-1)</td>
<td>200</td>
<td>438</td>
<td>260</td>
<td>0.59</td>
<td>–</td>
</tr>
<tr>
<td>25% PEG-400</td>
<td>18</td>
<td>5.38</td>
<td>160.2</td>
<td>29.77</td>
<td>50</td>
</tr>
<tr>
<td>5% PEG-6000</td>
<td>18</td>
<td>5.53</td>
<td>32.4</td>
<td>5.85</td>
<td>10</td>
</tr>
<tr>
<td>Crude (A26-2/11S)</td>
<td>200</td>
<td>333</td>
<td>310</td>
<td>0.93</td>
<td>–</td>
</tr>
<tr>
<td>25% PEG-400</td>
<td>30</td>
<td>9.0</td>
<td>243</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>5% PEG-6000</td>
<td>30</td>
<td>19.8</td>
<td>288</td>
<td>14.54</td>
<td>16</td>
</tr>
</tbody>
</table>
transucrase, which has a optimum pH in between 5.2–5.4.

Dextranucrase-producing (Gen Bank accession no. KC110687) Weissella cibaria JAG8 was isolated from apple by Jagan Mohan Rao and Goyal [18]. The optimum assay conditions for dextransucrase were 35°C, pH 5.4. The optimum pH's of the enzyme is quite different from that of our mutant strain's enzyme.

Analysis of dextranase hydrolyzates of dextrans prepared by new isolates

The structures of dextrans formed by AN39-1 and its mutants were studied by dextranucrase hydrolysis. Hydrolysis products of dextrans were analyzed by TLC (Fig. 3).

It is known that highly branched dextrans (B-1142, B-742, and B-1299) are resistant to endo-dextranase hydrolysis [19]. The dextrans produced by new enzymes were purified and hydrolyzed using *Penicillium dextranase* covalently immobilized onto Eupergit C. Soluble dextranase was not used in the analysis since it had invertase and glucose isomerase activities. However, the immobilized enzyme had only dextranase activity. TLC showed that the hydrolysis products (oligosaccharides) for all the dextrans produced by AN39-1 and its mutants were the same as the hydrolysis products of B-512F dextran, indicating that dextrans produced by AN39-1 and its mutants have mainly α-(1→6) linkages between glucose units (Fig. 3). Commercial dextran, produced by B-512F dextranucrase, has 95% α-(1→6) linkages and 5% α-(1→3) branch linkages. B-512 F
dextran is completely hydrolyzed by dextranase, yielding oligosaccharides. Dextranase hydrolysis products of dextrans synthesized by AN39-1 and its mutants were similar to those of commercial B-512 F dextran.

**Conclusion**

In the present study, mutant strains of AN 39-1 constitutive for dextransucrase were obtained to overcome problems of producing inducible enzyme. The mutant (A26-2/11), grown on glucose, produced 5.8 times more enzyme activities than the parent strain (AN39-1), grown on sucrose. Furthermore, enzyme produced by this mutant was more pH and temperature stable than the native enzyme. Dextran synthesized by the mutant enzyme was similar to commercial B-512 F dextran, possibly leading to be used in industrial applications.

**Conflict of Interest:** The authors have no conflict of interest.

**References**


