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Purification and characterization of a novel marine *Arthrobacter oxydans* KQ11 dextranase

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ABSTRACT

Dextranases can hydrolyze dextran deposits and have been used in the sugar industry. Microbial strains which produce dextranases for industrial use are chiefly molds, which present safety issues, and dextranase production from them is impractically long. Thus, marine bacteria to produce dextranases may overcome these problems. Crude dextranase was purified by a combination of ammonium sulfate fractionation and ion-exchange chromatography, and then the enzyme was characterized. The enzyme was 66.2 kDa with an optimal temperature of 50 °C and a pH of 7. The enzyme had greater than 60% activity at 60 °C for 1 h. Moreover, 10 mM Co^{2+} enhanced dextranase activity (196%), whereas Ni^{2+} and Fe^{3+} negatively affected activity. 0.02% xylitol and 1% alcohol enhanced activity (132.25% and 110.37%, respectively) whereas 0.05% SDS inhibited activity (14.07%). The thickness of *S. mutans* and mixed-species oral biofilm decreased from 54340 nm to 36670 nm and from 64260 nm to 43320 nm, respectively.

1. Introduction

Dextranase (α-D-1,6-Glucan-6-D-Gluca-nohydrolase, EC3.2.1.11) (Lee et al., 2010), hydrolyzes α-1,6 glucosidic dextran bonds (Bashari et al., 2013; Chen, Zhou, Fan, & Zhang, 2008; Eggleston & Monge, 2005; Finnegan, Brumbley, O'Shea, Nevalainen, & Bergquist, 2004; Khalikova, Susi, Usanov, & Korpela, 2003; Kim, Ko, Kang, & Kim, 2009; Larsson, Andersson, Stahlberg, Kenne, & Jones, 2003; Millson & Evans, 2007; Wu, Zhang, Huang, & Hu, 2011).

Many dextranases have been isolated from various microorganisms, such as mold (Arnold, Nguyen, & Mann, 1998; Fukumoto, Tsuji, & Tsuru, 1971; Goldstein-Lifschitz & Bauer, 1976; Hiraoka, Tsuji, Fukumoto, Yamamoto, & Tsuru, 1973; Pleszczynska, Rogalski, Szczodrak, & Fiedurek, 1996; Shimizu, Unno, Ohba, & Okada, 1998; Sugiura, Ito, Ogiso, Kato, & Asano, 1973), yeasts (Koenig & Day, 1989; Ryu et al., 2000) and bacteria (Arnold et al., 1998; Barrett, Barrett, & Curtiss, 1987; Chaiet et al., 1970; Fukumoto et al., 1971; Goldstein-Lifschitz & Bauer, 1976; Hiraoka, Fukumoto, & Tsuru, 1972; Hiraoka et al., 1973; Khalikova et al., 2003; Pleszczynska et al., 1996; Shimizu et al., 1998; Sugiura et al., 1973; Wynter et al., 1997), and these dextranases hydrolyzed dextran in an endo-wise or exo-wise fashion.

Dextranases are of practical importance: they can depolymerize various troublesome microbial dextran deposits. Thus, dextranase has been used in the sugar-production industry (Brown & Inkerman, 1992; Lee & Fox, 1985; Singleton, Horn, Bucke, & Adlard, 2001; Steels, Schoth, & Jensen, 2001) to remove dextran that is synthesized by diverse microorganisms and in oral hygiene to prevent dental caries (Finnegan et al., 2004; Larsson et al., 2003). Specifically, *Streptococci* produce an exo-polysaccharide composed predominantly of dextran (Khalikova et al., 2003), and dental plaques produced by *Streptococci mutans* and *S. sorbinus* can be removed by dextranases. Therefore, dextranases can be added to toothpaste or other dental products to prevent dental caries. Third, dextranase has been used to manufacture blood substitutes for some time (Eggleston & Monge, 2005; Khalikova et al., 2003; Kim & Day, 1994; Lee & Fox, 1985; Mehvar, 2000). Finally, dextranases are important for elucidating the structure of dextran and other polysaccharides (Cote, Ahlgren, & Smith, 1999; Ebisu, Misaki, Kato, & Kotani, 1974; Hayacibara et al., 2004).

Microbial strains which produce dextranase are chiefly mold, so safety issues preclude the use of mold to produce dextranase for the food industry. Also, the production time is unacceptably long.

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Marine bacteria that can produce dextranase may overcome these problems. The ocean is a high salt, low temperature, alkaline, environment, suggesting that enzymes from aquatic organisms may have unique catalytic properties and more potential applications compared to their terrestrial counterparts. Strains which isolated from the ocean to produce dextranase in our laboratory have low action temperatures, short production time (12–30 h), and stability under alkaline conditions. These properties suggest that marine *A. oxydans* KQ11 will have enormous economic and social benefits. Dextranase is an industrially used enzyme, therefore the easier the purification steps, the more widely it can be used.

Thus, the aim of the present work was to purify extracellular dextranase using a freeze-dried enzyme powder from *A. oxydans* KQ11 cultures using a rapid two-step method to estimate the feasibility of large-scale production as well as the characteristics of the enzyme produced.

2. Material and methods

2.1. Chemicals

Blue Dextran 2000, Dextran T-10, -40, -70, -500, and -2000 were all purchased from Solarbio. Dextran-20000 was purchased from Japan. Acrylamide, bis-acrylamide and SDS were the products of Sangon (Shanghai, China). Unstained Protein MW Marker was purchased from Thermo. Q Sepharose Fast Flow was purchased from GE Healthcare. All other reagents were of analytical grade.

2.2. Culture conditions for dextranase production

An *A. oxydans* KQ11 (GenBank sequence accession number JX481352.1) which could secrete dextranase was cultivated in medium containing 0.75% soymeal, 0.75% cassava starch, 0.5% yeast extract powder, 1% wheat bran, 0.4% Dextran 20000, 0.4% NaCl and 0.04% $MgSO_4$ (pH 7.5). After 30 h of aerobic fermentation at 30 °C, 150 rpm, the thallus of the fermentation broth was removed with a diatomite filter, and liquid supernatant was filtrated using an ultrafiltrate membrane and freeze-dried. Enzymes used in the purification experiment came from the freeze-dried enzyme powder.

2.3. Dextranase activity assay

Dextranase activity was measured by the method of Miller using the increased ratio of the reducing sugar concentration in the reaction with 3,5-dinitrosalicylic acid reagent (Eggleston & Monge, 2005; Hild, Brumbley, O'Shea, Nevalainen & Bergquist, 2007; Wang, Wang, He, & Zhang, 2012). A mixture of dextranase (0.01 mL) and 0.19 mL of 50 mM sodium phosphate buffer, pH 7, was incubated at 50 °C for 15 min and DNS (Lever, 1972) was added to the experimental and control groups to terminate the reaction. Then, 0.01 mL enzyme was added to the control group. Finally, the mixture was boiled for 5 min and 3 mL distilled water was added. The absorbance of the mixture was measured at 540 nm. One unit of dextranase activity (U) was defined as the amount of enzyme that catalyzed the liberation of 1 μmol isomaltose (measured as maltose) in 1 min from Dextran 20000 (Smogyi, 1952).

2.4. Protein measurement

Protein (mg/mL) was measured with the Bradford method (Bradford, 1976; Zor & Seliger, 1996) using crystalline bovine serum albumin as the protein standard. Moderate pure dextranase was mixed with distilled water (total volume = 1 mL) and 3 mL Coomassie brilliant blue G250 was added to the test tube containing the mixture described above and all was blended well. The absorbance was observed at 595 nm and the protein concentration was calculated.

2.5. Enzyme purification

2.5.1. Ammonium sulfate fractionation

Ammonium sulfate fractionation was used to purify protein (Chaiet et al., 1970; Igarashi, Yamamoto, & Goto, 1992; Wynter et al., 1997). Enzyme powder obtained from the fermentation test was dissolved in ultrapure water (2.5 g/100 mL) and solid $(NH_4)_2SO_4$ (36.1 g/100 mL) was added on a magnetic stirrer at room temperature and kept at 4 °C for 4 h. Then the solution was centrifuged at 12,000 rpm for 20 min. The liquid supernatant was collected by a micropipette and solid $(NH_4)_2SO_4$ (6.2 g/100 mL) was added on a magnetic stirrer at room temperature and the solution was kept at 4 °C for 4 h. Then, precipitate was collected, and re-suspended in 20 mM Tris–HCl buffer (pH 7.5, 15 mL).

2.5.2. Ion-exchange chromatography on Q Sepharose Fast Flow

Chromatography was performed using Bio-Rad Biologic Duo-Flow chromatographic system. A Q Sepharose Fast Flow column (10 cm × 2 cm) was equilibrated with 20 mM Tris–HCl buffer (pH 7.5) for 1 h until the baseline was smooth and pH 7.5. The enzyme was eluted from column with a 30% NaCl gradient, at a flow rate of 1 mL/min for 90 min. Fractions containing substantial dextranase activity were collected with centrifugal filter units (Millipore, city, state).

2.5.3. SDS-PAGE

Protein samples and unstained protein marker were separated using 10% w/v SDS-PAGE with 0.5% Blue Dextran (BD-SDS-PAGE) according to Laemmli (Laemmli, 1970), using a PowerPac HV (BioRad, city, state). The gel was washed with distilled water and incubated at room temperature for 30 min in 20 mM Tris–HCl, pH 7.5 (Khalikova et al., 2003), containing 2.5% Triton X-100, followed by a 3 h incubation at 37 °C in 20 mM Tris–HCl, pH 7.5 until a white band appeared.

3. Results and discussion

3.1. Summary of purification for dextranase from A. oxydans KQ11

Total protein and enzyme activity as well as the yield of purified dextranase from *A. oxydans* KQ11 is shown in Table 1. The result showed that the final purification fold and yield of the dextranase

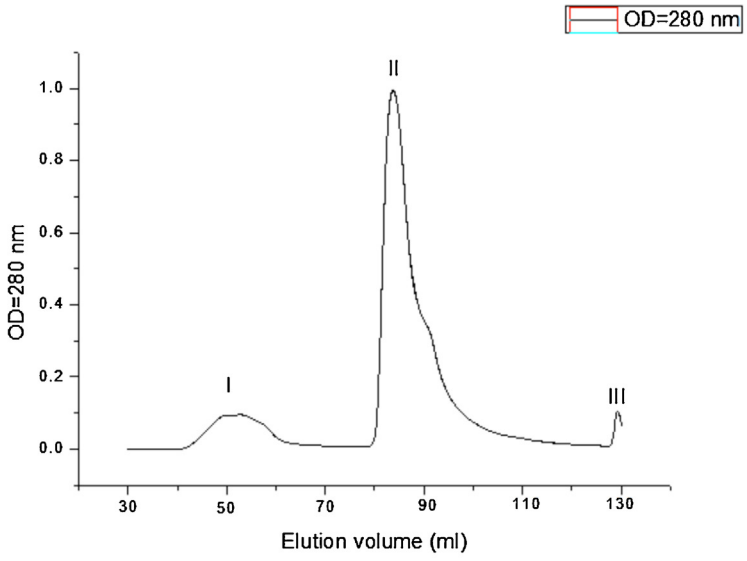
Table 1
Summary of the purification of dextranase from *A. oxydans* KQ11.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yields (%)
Crude enzyme	318.81	269.72	0.85	1	100
Ammonium sulfate precipitation	21.05	144.65	6.87	8.12	53.63
Ion-exchange chromatography on Q Sepharose Fast Flow	2.81	102.21	36.38	43.00	37.90

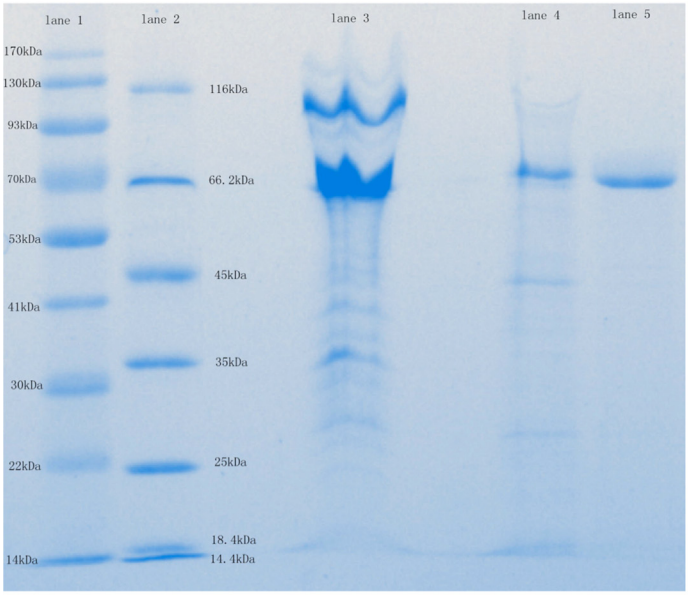
were 43.00% and 37.90%, respectively, which was obviously 35-fold and 27% better than the counterparts mentioned in published methods (Wu et al., 2011) using a similar two-step purification. Dextranase had a single peak with ion-exchange chromatography (Fig. 1a). SDS-PAGE (Fig. 1b) and BD-SDS-PAGE (Fig. 1c) revealed a molecular weight of pure dextranase of ~66.2 kDa, similar to dextranase from *Thermotoga lettingae* (Kim & Kim, 2010) and a recombinant *Lipomyces starkey* dextranase (Chen et al., 2008). No other bands appeared, so the purification was successful.

3.2. Effect of temperature and pH on enzyme activity and stability

To determine the optimal temperature, dextranase activity was measured at temperatures ranging from 10 to 70 °C, at pH 5.5. The optimal temperature for dextranase was 50 °C and at this temperature, dextranase activity was greatest. To measured thermal stability, dextranase activity was measured at 30, 40, 50, and 60 °C, respectively and more than 80% residual activity was retained after 5 h of incubation at 30 and 40 °C. The enzyme



(a)



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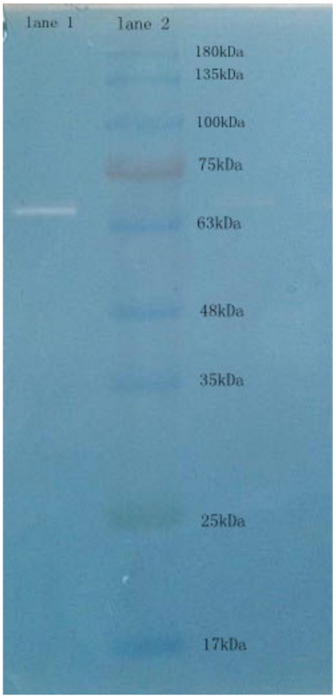

(c)

Fig. 1. (a) Ion-exchange chromatography from the Bio-Rad Biologic DuoFlow chromatographic system. (b) Result of pure dextranase collected from ion-exchange chromatography with 15% SDS-PAGE. Lane 1: prestained protein marker, lane 2: unstained protein marker, lane 3: crude enzyme, lane 4: enzyme collected after ammonium sulfate fractionation, lane 5: purified dextranase (Fraction II, 20 μl containing 28 μg protein). (c) BD-SDS-polyacrylamide electrophoresis was performed in 15% acrylamide gel. Lane 1: purified dextranase (Fraction II, 2 μl containing 2.8 μg protein), lane 2: prestained protein maker.

maintained more than 60% activity at 60 °C for 1 h, which may be useful for high-temperature sugar milling processes. Thus, the optimal catalytic and thermostable temperature of pure dextranase was 50 °C and 30 °C, respectively.

The effect of pH on dextranase activity was assayed at pHs from 3 to 9 at 50 °C, and the optimal pH was 7. Various buffers used were acetate, phosphate and Tris–HCl buffer, pH of which was 3–6, 6–8 and 7–9, respectively. Data revealed more than 80% residual activity was retained after 1 h of incubation at room temperature at a pH of 7.5 and 8 and these were concluded to be the optimal catalytic and stable pHs. These data can be applied to the sugar milling and oral hygiene industries to improve products.

3.3. Effect of metal ions on dextranase from A. oxydans KQ11

The effect of metal ions (K^+, NH^{4+}, Li^+, Cd^{2+}, Si^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}) and other reagents ($ZnCl_2$, SLS, xylitol, benzoic acid, SDS, menthol, sodium fluoride, $ZnSO_4$, potassium thiocyanate, alcohol) on dextranase activity were assayed by treating dextranase with 1, 5, and 10 mM metal ions at pH 7 for 3 h at 37 °C (Chen et al., 2008), and measuring enzyme activity (Table 2). The final concentration of these reagents were 0.02% $ZnCl_2$, 0.02% SLS, 0.02% xylitol, 0.02% benzoic acid, 0.05% SDS, 0.05% menthol, 0.05% sodium fluoride, 0.05% $ZnSO_4$, 0.05% potassium thiocyanate, and 1% alcohol. Data from these experiments are shown in Table 2.

The result showed that when the final concentration of these metal ions were 1 mM, 5 mM, 10 mM, respectively, relative activity was greater than 85% with Li^+, Ca^{2+} and Mg^{2+} at 1 mM, K^+, Si^{2+}, Ca^{2+} and Co^{2+} at 5 mM, K^+, NH_4^+, Li^+, Si^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+} and Co^{2+} at 10 mM. In contrast, relative activity was below 25% with Ni^{2+} and Fe^{3+} (Chen et al., 2008; Kim & Kim, 2010) at 1, 5, and 10 mM. Ca^{2+} had positive effect on pure dextranase activity, while Ni^{2+} and Fe^{3+} had negative effects, and 10 mM Co^{2+} enhanced dextranase activity 196%. These data will provide a biological basis for the application of dextranase from *A. oxydans* KQ11 in toothpaste and gargle.

3.4. Enzyme kinetics

To determine the kinetic constants (Wu et al., 2011), the initial velocity (v) was measured with different concentrations of T-40, T-70, and T-2000 (0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0% w/v) in 0.05 M phosphate buffer (pH 7.0) at 25 °C. Then, kinetic constants were calculated from Lineweaver–Burk plots. Data show that the affinity between dextran T-40 and dextranase was the greatest, whereas dextran T-2000 had the least affinity for dextran.

Table 2
Effect of metal ion on dextranase from *A. oxydans* KQ11.

Metal ions	Relative activity (%) (1 mM)	Relative activity (%) (5 mM)	Relative activity (%) (10 mM)
Control	100 ± 0.288	100 ± 0.288	100 ± 0.288
K^+	82.24 ± 0.232	94.52 ± 0.271	104.09 ± 0.301
NH_4^+	84.38 ± 0.239	68.23 ± 0.190	94.98 ± 0.275
Li^+	93.25 ± 0.267	83.22 ± 0.237	104.32 ± 0.302
Cd^{2+}	79.65 ± 0.224	54.11 ± 0.144	74.00 ± 0.209
Si^{2+}	82.76 ± 0.233	86.05 ± 0.244	106.00 ± 0.308
Ni^{2+}	8.15 ± 0.019	6.13 ± 0.014	8.10 ± 0.010
Ca^{2+}	89.38 ± 0.255	90.03 ± 0.256	104.67 ± 0.308
Cu^{2+}	70.88 ± 0.201	53.64 ± 0.142	25.28 ± 0.065
Ba^{2+}	82.03 ± 0.228	75.55 ± 0.211	89.51 ± 0.255
Mg^{2+}	93.48 ± 0.268	77.23 ± 0.216	86.10 ± 0.249
Zn^{2+}	71.46 ± 0.208	54.16 ± 0.146	48.86 ± 0.127
Co^{2+}	74.80 ± 0.219	124.27 ± 0.365	196.86 ± 0.595
Fe^{3+}	9.19 ± 0.010	5.04 ± 0.045	14.61 ± 0.028

3.5. Substrate specificity of dextranase from A. oxydans KQ11

Substrate specificity of dextranase was studied using a hydrolysis experiment, different dextrans, soluble starch, pullulan and microcrystalline cellulose was used as a substrate. All substrates, except soluble starch consisted of α-1,4, and α-1,6 glucosidic bonds and pullulan, microcrystalline cellulose consisted of α-1,4 glucosidic bonds (the others contained α-1,6 glucosidic bonds). Data from substrate specificity experiments with *A. oxydans* KQ11 dextranase showed that the enzyme hydrolyzed α-1,6 glycosidic bonds and not α-1,4 glucosidic bonds (Table 3).

3.6. Effect of mouthwash on A. oxydans KQ11 dextranase

Data in Fig. 2 show that 0.02% xylitol and 1% alcohol promoted enzyme activity whereas 0.05% SDS (Chen et al., 2008), 0.05% Menthol and 0.05% $ZnSO_4$ inhibited the activity. 0.02% $ZnCl_2$, 0.02% SLS, 0.02% sodium benzoate, 0.05% NaF and 0.05% potassium thiocyanate had no significant effect on enzyme activity.

3.7. Effect of dextranase from A. oxydans KQ11 on thickness of Streptococcus mutans biofilm and mixed-species oral biofilm

2% *S. mutans* was inoculated into 50 mL BHI culture medium and was cultured at 37 °C and under strictly anaerobic conditions for 18 h. Then the thallus was collected through centrifugation at 10,000 rpm, 4 °C and washed with normal saline for 1-times. The thallus was suspended and the final concentration of *S. mutans* was adjusted to OD_{550} = 1.0 with normal saline. Coverslips were placed in a 4-hole plate containing BHI culture medium and 1% sugar, the suspended thallus was inoculated. After 4 h, the biofilm formed and the experimental group was treated with pure dextranase while the control group was not treated. Then all these coverslips were washed with normal saline for times and BHI culture medium was renewed until these coverslips were taken out 5 days later. The thickness of biofilm was observed with confocal laser scanning microscopy (CLSM).

The preparation method of mixed-species dental biofilm was same as mentioned above, the difference was that the components of mixed-species oral biofilm were *Streptococcus mutans*, *S. salivarius*, *Streptococcus sanguis*, *Latobacillus* and *Actinomyces viscosus*. The ratio of them was 1:1:1:1:1.

The result showed that dextranase contributed to the degradation of *S. mutans* biofilm (Fig. 3a and Fig. 3b) and mixed-species oral biofilm (Fig. 3c and Fig. 3d). The thickness of *S. mutans* biofilm (Duarte et al., 2006) was decreased from 54340 nm to 36670 nm and the thickness of mixed-species oral biofilm (Jeon et al., 2011) was decreased from 64260 nm to 43320 nm. This experiment provided a theoretical foundation for the application of dextranase in the caries prevention.

Table 3
Substrate specificity of dextranase from *Arthrobacter oxydans* KQ11.

Substrate	Main linkage	Relative activity (%)
Dextran T-10	a-1, 6	98.88 ± 1.168
Dextran 20000	a-1, 6	91.31 ± 1.077
Dextran T-40	a-1, 6	100 ± 1.181
Dextran T-70	a-1, 6	98.30 ± 1.162
Dextran T-500	a-1, 6	85.11 ± 1.001
Dextran T-2000	a-1, 6	86.69 ± 1.023
Soluble starch	a-1, 4, a-1, 6	2.50 ± 0.021
Pullulan	a-1, 4	0
Microcrystalline cellulose	a-1, 4	0

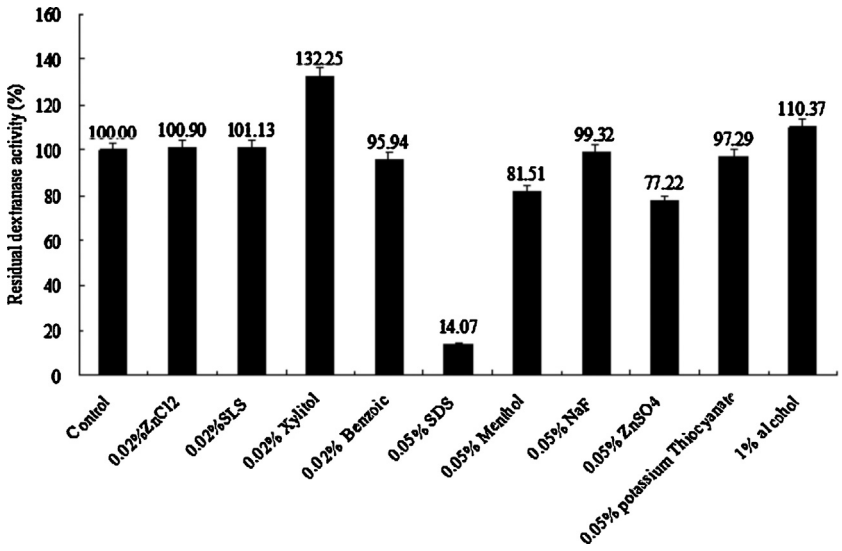

Fig. 2. Effect of mouthwash effective ingredients on dextranase from *A. oxydans* KQ11.

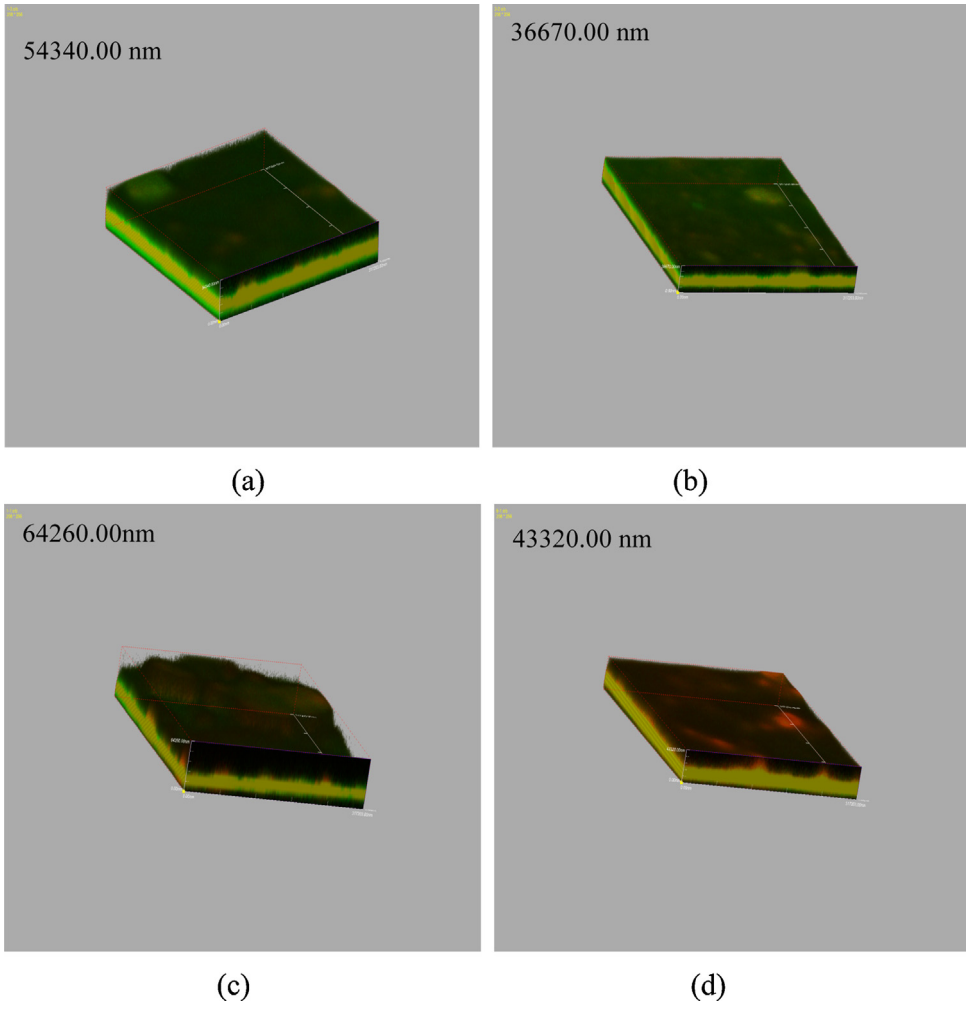

Fig. 3. Effects of dextranase on thickness of *S. mutans* biofilm and mixed-species dental biofilm.

4. Conclusions

Dextranase from a marine *A. oxydans* KQ11 was purified by combining ammonium sulfate precipitation and ion-exchange chromatography with Q Sepharose Fast Flow. The enzyme had a single peak on ion-exchange chromatography and a single band appeared after SDS-PAGE and BD-SDS-PAGE. Dextranase had an optimal pH of 7.0 and temperature of 50 °C (pH 7.0). The thermostable temperature and stable pH of pure dextranase was 30 °C and 8, respectively. Ca^{2+} positively affected pure dextranase activity, whereas Ni^{2+} and Fe^{3+} had negative effects. The affinity between dextran T-40 and dextranase was the greatest, whereas dextran T-2000 had the least affinity. The enzyme hydrolyzed α-1,6 glucosidic bonds and not α-1,4 glucosidic bonds. Both 0.02% xylitol and 1% alcohol promoted enzyme activity whereas 0.05% SDS inhibited the enzyme. No other reagents significantly affected the enzyme. It had more than 60% activity at 60 °C for 1 h, which may be useful for high-temperature sugar milling processes. The thickness of *S. mutans* biofilm and mixed-species oral biofilm treated with purified dextranase was decreased from 54340 nm to 36670 nm and from 64260 nm to 43320 nm respectively, which provided a theoretical foundation for the application of dextranase in the caries prevention. These characterizations will enable future use of dextranase from marine *Arthrobacter oxydans* KQ11 in the food industry and lay the foundation for future enzymatic studies.

Acknowledgements

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