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ORIGINAL ARTICLE

Screening, production, and characterization of dextranase from *Catenovulum* sp.

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Abstract A psychrotolerant dextranase-producing bacterium was isolated from the Gaogong island seacoast near Jiangsu, China. The bacterium, denoted as DP03, was identified as *Catenovulum* sp. based on its phenotype, biochemical characteristics, and 16S rRNA gene comparison. The optimal enzyme production time, initial pH, temperature, and aeration conditions of strain DP03 were found to be 28 h, 8.0, 30 °C, and 25 % volume of liquid in 100-ml Erlenmeyer flasks, respectively. The ability of 1 % dextran T20 to induce dextranase was investigated. Dextranase from strain DP03 displayed its maximum activity at pH 8.0 and 40 °C and was found to be stable at 30 °C and over a broad range of pH values (pH 6–11). Scanning electron microscopy showed that dextranase from the isolate DP03 could at least partially prevent *Streptococcus mutans* from forming biofilms on glass coverslips.

Keywords *Catenovulum* · Dextranase · Dextran · Fermentation · Enzyme production · Biofilm

Introduction

Dextranases (α-1,6-D-glucan-6-glucanohydrolase; EC 3.2.1.11) catalyze the endohydrolysis of α-1,6-linkages of dextrans to oligosaccharides and are widely used in the sugar processing industry, medicine, and dentistry. In commercial sugar processing, the use of dextranase in the degradation of dextran has been shown to be the most suitable method of solving the problems of poor clarification and throughput in sugarcane juice, both of which are caused by the presence of dextran (Bowler and Wones 2011; Park et al. 2012; Purushe et al. 2012). In medicine, pathogen-free solutions of dextran of a specific molecular weight can be used as blood alternatives during emergencies (Mehvar 2000), while in dentistry the possibility of dextranases being capable of degrading the oral biofilm (commonly known as dental plaque) has focused the attention of researchers on the use of these enzymes in the treatment of dental plaques (Keyes et al. 1971). According to Marotta et al. (2002), dental plaques contain a considerable amount of dextran which can be degraded by dextranase. Fungi are the most common source of dextranase. They produce many acidic and high-temperature catalytic enzymes that are capable of catalyzing reactions at pH values ranging from 5 to 6.5 and temperatures of >50 °C; they tend, however, to be unstable under alkaline conditions. Those dextranases derived from fungi are rarely effective under weakly alkaline conditions and lower temperature (Khalikova et al. 2005).

Marine microorganisms and enzymes may be an efficient solution to this problem because of the cold and alkaline environments in which they are found (Papaleo et al. 2011). Cold-adapted (cold-active) enzymes have an enormous biotechnological potential in fields such as detergent formulation, the sugar and dairy industries, environmental biosensors, and bioremediation (Cieslinski et al. 2005). Some cold-adapted enzymes derived from marine microbes may serve as sources of novel medicines (Gulder and Moore 2009; Kalpana et al. 2012). Alkaline dextranase may play an important role in the treatment of oral dental plaques, since alkaline tooth-rinse products are expected to be more amicable to enamel compared to acidic ones (Majeed et al. 2011). In the study reported here, we isolated and identified a dextranase-producing

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marine bacterium. The culture conditions for dextranase production and the crude enzyme properties were subsequently investigated.

Materials and methods

Samples and chemicals

Sea water samples were collected from the Gaogong island seacoast in Jiangsu (China). Dextran (T20, T40, T70, T500) and blue dextran 2000 were obtained from GE Healthcare (Uppsala, Sweden). Electrophoresis-pure grade dextranase from *Penicillium janthinellum* was purchased from Sigma-Aldrich (St. Louis, Mo). All other reagents were purchased from Sinopharm Chemical Reagent Corp. (Shanghai, China) and were of the highest analytical grade.

Isolation and characterization of the extracellular-dextranase-producing bacterial strain

Screening of marine bacteria for dextranase production was carried out using a blue dextran plate method. The medium contained 1 g yeast extract, 5 g peptone, 8 g dextran, 2 g blue dextran 2000, 20 g agar powder, and 1 l natural sea water and had an initial pH of 7.8. The isolate that produced the highest ratio of transparent zone to colony diameter was chosen for further study.

The method described by Lu et al. (2010) was used to determine the optimal growth temperature, pH, and NaCl concentration conditions for growth; phenotypic and biochemical characterization of strain DP03 was also carried out. To determine the optimal growth temperature, cells were cultivated at different temperatures ranging from 15 to 35 °C. The optimal pH and NaCl concentration for growth were determined at 30 °C. To determine the growth rates at different pH, cells were cultivated in growth media at different pH (range 4–11). To determine the optimal NaCl concentration, cells were cultured in growth media containing 0–11 % NaCl. All experiments were performed in triplicate. The 16S rDNA sequence of the strain DP03 was also identified from the genomic DNA and amplified by PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplified product was then purified and sequenced. The nucleotide sequence has been submitted to GenBank under accession No. JX276658. A similarity search was performed using the GenBank database and the Clustal W program (Thompson et al. 1994).

Culture conditions for dextranase production

The liquid culture medium for the extracellular production of dextranase (the production medium) contained 5 g/l yeast extract, 5 g/l peptone, 10 g/l dextran T20, and 5 g/l NaCl. The mixture had an initial pH of 8.0 before autoclaving. A 0.5-ml aliquot of pre-inoculum was inoculated into 50 ml of same liquid culture medium (in 250-ml Erlenmeyer-type flask), and the mixture was incubated on a rotary shaker at 200 rpm and 30 °C for 28 h, following which the cultured cells were separated by centrifugation (10 min, 10,000 *g*, 4 °C) and the supernatant used for dextranase activity determination.

Enzyme assay

Enzyme activity was measured using dextran T70 as substrate (3 %, m/v) in 0.1 M Tris–HCl buffer (pH 7.5) using

Table 1 Morphological, physiological, and biochemical characteristics of strain DP03 (*Catenovulum* sp.)

Characteristics	Value
Morphological characteristics	
Shape	Rod
Colony color	Pale yellow
Physiological and biochemical characteristics	
Gram stain	−
Spore	−
Motility	+
Growth at:	
4 °C	−
37 °C	+
NaCl range for growth (w/v, %)	0.5–7
Arginine dihydrolase	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
ß-Glucosidase	+
ß-Glucuronidase	+
Malonate	−
Lipase	+
Oxidase	+
Indole	−
Methyl red test	+
Vopes and Prokauer test	−
Urease	−
Mannitol	−
Glucose	+
Trehalose	−
Cellobiose	+
Maltose	+
Arabinose	+
Sucrose	+
Arabitol	−
Galacturonate	−

the 3,5-dinitrosalicylic acid method. Maltose served as the standard. One unit of dextranase activity was defined as the amount of enzyme capable of hydrolyzing dextran to 1 μmol of reducing sugar in 1 min (Hild et al. 2007).

Effects of time, initial pH, and temperature on dextranase production

The optimal culture time for dextranase production was within the range of 0 and 48 h, and samples were collected at 2-h intervals. To determine the optimal initial pH for dextranase production, the production medium was adjusted to different initial pH values (range 4–10) and incubated at 30 °C for 28 h. The effects of temperature on dextranase production were assessed by culturing the DP03 strain at temperatures ranging from 15 to 35 °C for 28 h at pH 8.0. Subsequent experiments were then performed under the determined optimal initial pH and temperature conditions.

Effects of aeration on dextranase production

The effects of aeration on dextranase production were investigated in 100-ml Erlenmeyer-type flasks containing 15, 20, 25, 30, 35, 40, and 45 ml of the production medium, as described previously.

Effects of carbon and nitrogen source on dextranase production

Different carbon sources (5 g/l; dextrin, cellulose, potato starch, corn starch, cassava starch, lactose, glucose, and starch sugar) and nitrogen sources (5 g/l; ammonium sulfate, ammonium nitrate, peptone, yeast extract, soybean powder, milled corn, and casein) were chosen to replace yeast extract

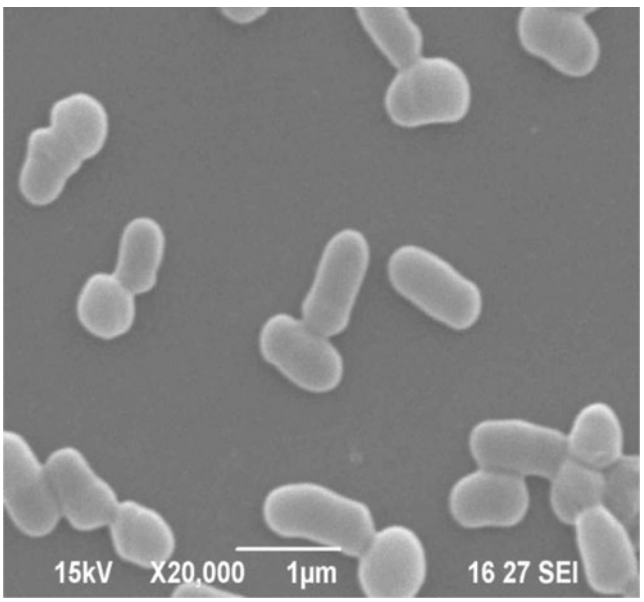

Fig. 1 Scanning electron micrograph of strain DP03 (*Catenovulum* sp.)

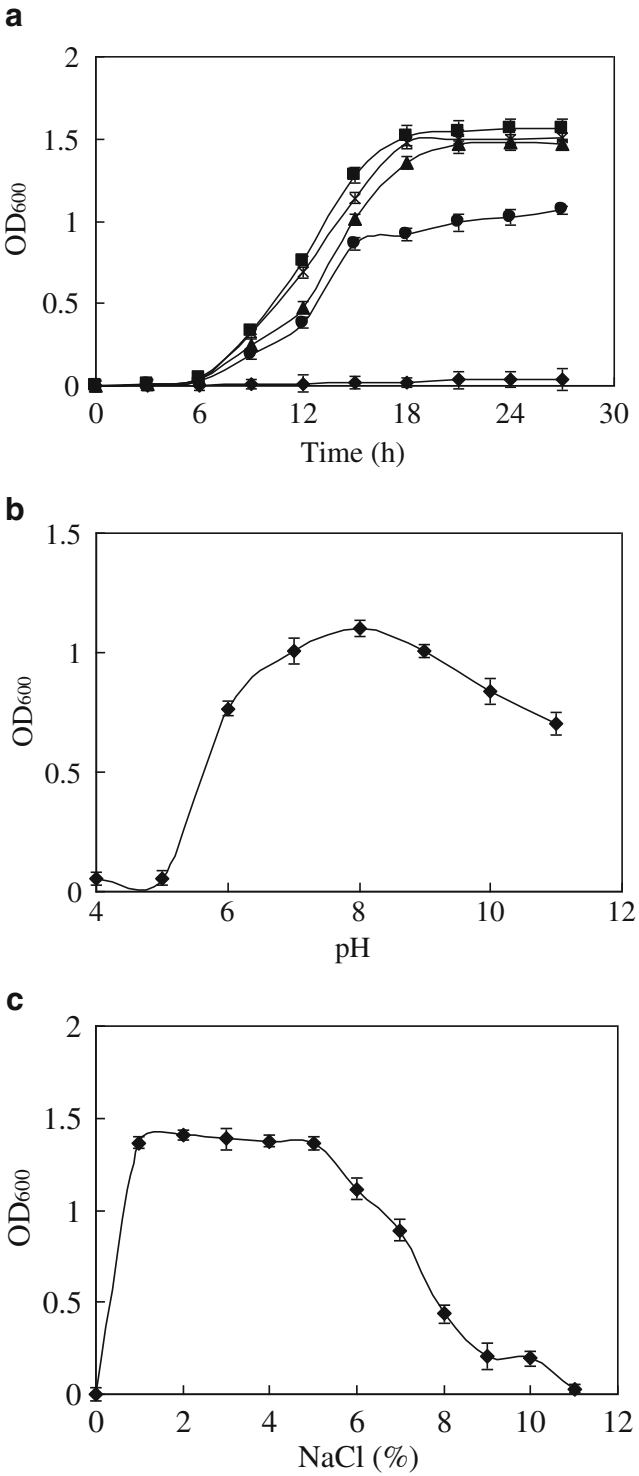

Fig. 2 Effects of temperature (**a**; *filled diamond* 15 °C, *filled circle* 20 °C, *filled triangle* 25 °C, *filled square* 30 °C, × 35 °C), initial pH (**b**), and NaCl concentration (**c**) on the growth of strain DP03 (*Catenovulum* sp.)

and peptone in the production medium. The effects of these different carbon and nitrogen sources on dextranase production under optimal initial pH and temperature conditions were examined.

Optimal type of dextran and effects of dextran concentration on dextranase production

Different types of dextran (dextran T20, T40, T70, and T500) were tested in order to determine their effect on dextranase production. Each type of dextran was tested at various concentrations (0–1.4 %), and the chose concentration was added to the production medium as a replacement for dextran.

Effects of pH on enzyme activity and stability

The enzyme was used for measuring its activity at different pH values (in 50 mM buffers) and at 40 °C. All buffers contained 3 % dextran T70. To determine the pH effect on the stability of dextranase, the enzyme was incubated at 25 °C for 1 h, following which the residual activity was measured. Solutions (50 mM) with different pH values were: citrate buffer (pH 4–6), sodium phosphate buffer (pH 6–7.5), Tris–HCl (pH 7.5–9.0), and $NaHCO_3$–Na_2CO_3 (pH 9.0–11.0). All measurements were processed at 37 °C.

Effects of temperature on enzyme activity and stability

The enzymatic function was evaluated at different temperatures ranging from 0 to 55 °C at pH 8.0. To assess thermal stability, the enzyme production supernatant was pre-heated at 30 °C, 40 °C, and 50 °C for 1–5 h. Residual enzymatic activity was assessed at pH 8.0.

Preliminary assessment of the ability of dextranase to prevent biofilm formation

The effects of dextranase on biofilm produced by *Streptococcus mutans* ATCC 25175 were investigated using scanning electron microscopy (SEM). *S. mutans* was first precultured in a brain heart infusion (BHI) medium without sucrose at 37 °C for 15 h, and then 1 ml of this precultured solution was inoculated into a new BHI medium with 1 % sucrose (20 ml in 100-ml flask). Sterile glass coverslips were placed on the BHI medium. The media were co-cultured with *S. mutans* and a crude extract containing *Catenovulum* dextranase (40 units, cell-free) from strain DP03 at 37 °C for 24 h. Three identical assays with an equal volume of cell-free pure water, thermal inactivated *Catenovulum* dextranase, and a *Penicillium janthinellum* dextranase (40 units, cell-free) served as the blank control, negative control, and positive control, respectively. All coverslips were collected for fixation. They were first dehydrated and dried according to the procedure described by Tao et al. (2011), following which the coverslips were sputter-coated with gold (model JFC-1600, JEOL, Tokyo, Japan) and viewed using by SEM (model JSM-6390LA; JEOL, Tokyo, Japan).

Results

Screening of the strain producing extracellular dextranase

The strain producing extracellular dextranase was found to produce a transparent zone in the blue dextran plate (see supplementary material). Of 80 strains tested, four were found to produce extracellular dextranase. Of these, we selected the strain with the largest ratio of the diameter of the transparent area to the diameter of the colony (3.4 cm) for further testing.

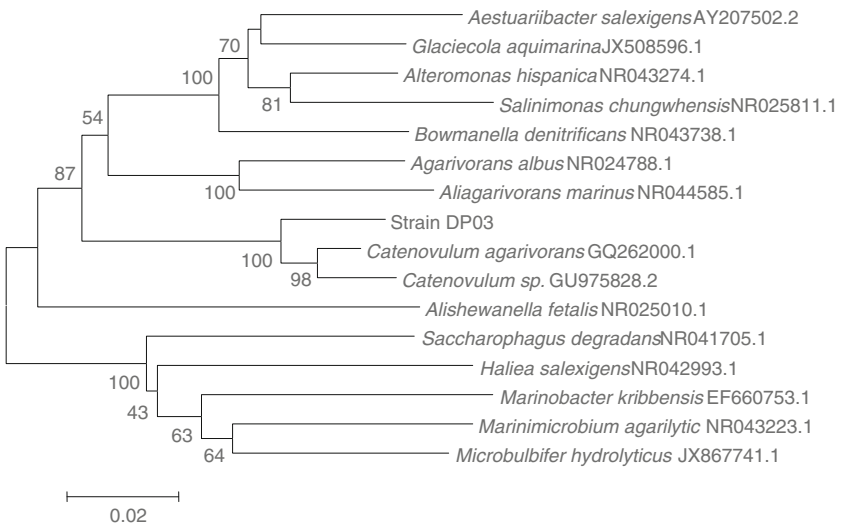

Fig. 3 Phylogenetic tree based on 16S rRNA gene sequences

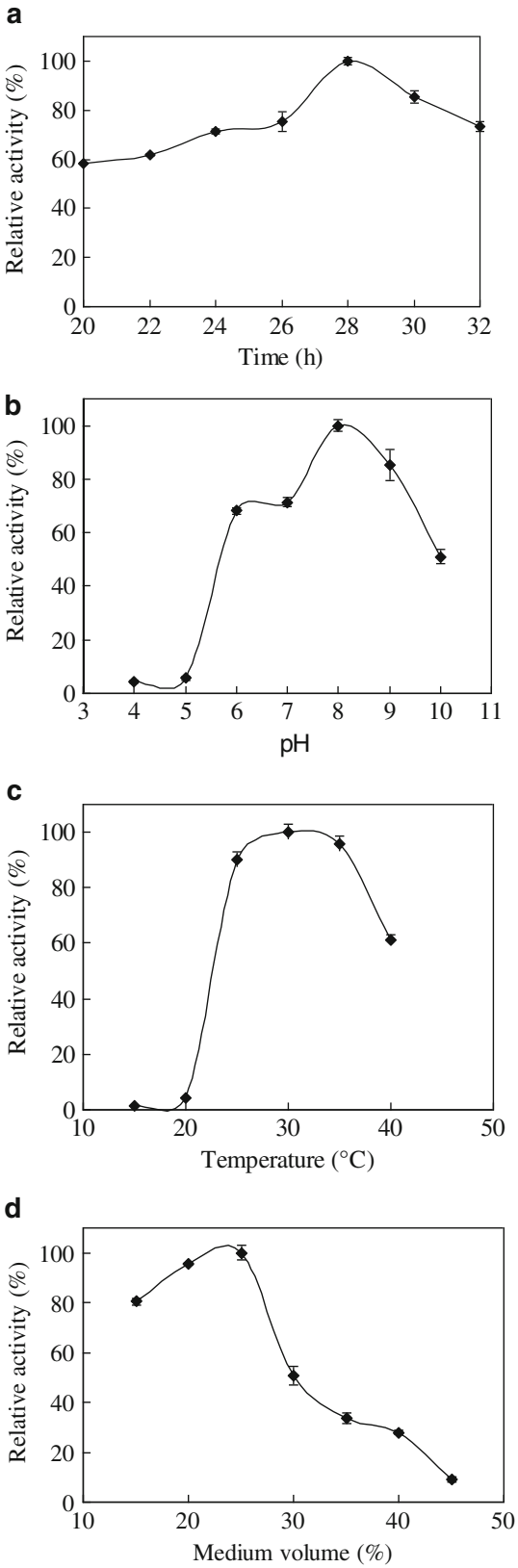

Fig. 4 Effects of time (**a**), initial pH (**b**), temperature (**c**), and aeration (**d**) on dextranase production. For each effect on dextranase activity, the values are shown as percentages of the maximum activities, which were taken as 100 %

The selected strain, which showed a dextranase activity of 17.3 U/ml as determined by the dextranase assay, was denoted strain DP03.

Identification of strain DP03

The morphological and biochemical characteristics of strain DP03 are listed in Table 1. This strai was found to be a Gram-negative, aerobic, and rod-shaped bacterium (0.3–0.5×0.7–1.0 μm) (Fig. 1) capable of growing at temperatures up to 37 °C but not capable of growing at 4 °C. Optimal growth was observed at 30 °C (Fig. 2a). Strain DP03 was found to grow at a pH range of 6–11 with an optimum near pH 8.0 (Fig. 2b). Growth occurred in media containing 1–6 % NaCl, with the maximum growth rate observed to be around 2 % NaCl. The strain could not grow in the absence of NaCl (Fig. 2c).

Sequencing of the 16S rRNA gene of strain DP03 was performed. Its 16S rDNA sequence was aligned using 16S rRNA gene sequences retrieved from GenBank to calculate a phylogenetic tree using MEGA software ver. 5.0 and ClustalX ver. 1.83. Based on other species of the family *Alteromonadaceae*, the phylogenetic tree indicates that strain DP03 belongs to genus *Catenovulum* (Fig. 3). On the basis of morphological and biochemical characterization, in combination with 16S rRNA gene sequence analysis, the selected strain was identified as *Catenovulum* sp. and denoted *Catenovulum* sp. DP03.

Dextranase production conditions

Effects of time, initial pH, temperature, and aeration on dextranase production

The effects of time on production of dextranase are given in Fig. 4a, which shows that the cultivation time required for maximal dextranase production was 28 h (based on 2-h sampling intervals). The effects of the initial pH of the medium are shown in Fig. 4b. When the initial pH value of the growth medium was adjusted over a range of 4.0 to 10.0, optimal enzyme production was observed at pH 8.0. Temperature was found to influence dextranase production. All temperature experiments (range tested: 15–40 °C) were carried out at pH 8.0, and higher enzyme production was observed at 30 °C (Fig. 4c). The influence of aeration on dextranase production is shown in Fig. 4d; the optimum aeration rate of dextranase production was obtained using a 100-ml Erlenmeyer-type flask with 25 ml of culture.

Effects of carbon and nitrogen source on dextranase production

The effects of different carbon sources on dextranase production is presented in Table 2. Potato starch was the best carbon source followed by cassava starch and cellulose for

Table 2 Effects of carbon and nitrogen sources on dextranase production

Carbon source (5 g/l)	Dextranase activity (U/ml)	Nitrogen source (5 g/l)	Dextranase activity (U/ml)
Dextrin	8.92±0.12	Ammonium sulfate	15.66±0.32
Cellulose	13.95±0.28	Ammonium nitrate	13.24±0.31
Potato starch	19.02±0.34	Peptone	18.95±0.36
Corn starch	15.69±0.21	Yeast extract	17.79±0.27
Cassava starch	18.88±0.28	Soybean powder	16.15±0.22
Lactose	11.90±0.14	Milled corn	6.28±0.23
Glucose	9.94±0.11	Casein	0
Starch sugar	1.28±0.06		

dextranase production. Dextrin, lactose, glucose, and starch sugar caused a sharp decrease in enzyme production.

With respect to the influence of nitrogen sources on the production of dextranase, Table 2 shows that based on measurements of enzymatic activity, yeast extract and peptone caused remarkably high levels of dextranase production, while soybean powder, corn meal, and casein decreased the production of dextranase. The use of an inorganic nitrogen source, such as ammonium sulfate and ammonium nitrate, also had an effect on dextranase production.

Effects of the presence and concentration of optimal inducers on the induction of dextranase production

Dextranase from strain DP03 was found to be a dextran-inducible enzyme. The optimal inducer was dextran T20, and maximum activity was observed in the presence of 1 % dextran T20 (Fig. 5). The results also showed that lower-molecular-weight dextran was a more effective inducer than higher-molecular-weight dextran.

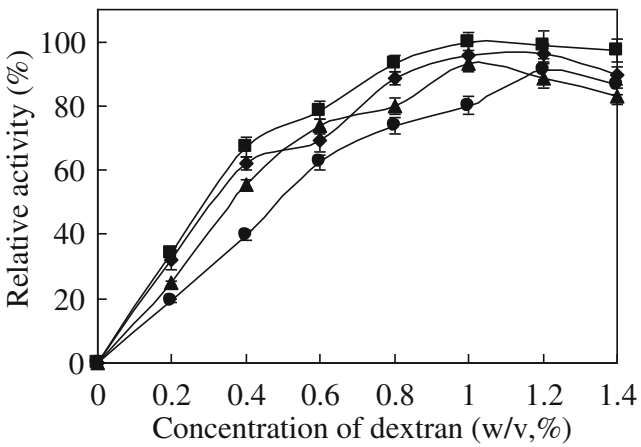

Fig. 5 Effects of different types of dextran on dextranase production. *Filled square* Dextran T20, *filled diamond* dextran T40, *filled triangle* dextran T70, *filled circle* dextran T500. For each effect of dextran on dextranase activity, the values are shown as percentages of the maximum activities, which were taken to be 100 %

Enzymatic characterization

Effects of pH on enzyme activity and stability

The optimum pH for the maximum dextranase activity was found to be 8.0, as measured using crude enzyme (Fig. 6). Figure 6 also shows that dextranase from strain DP03 retained more than 60 % of its activity within a pH range of 6–11 (100 % at pH 7.0) and that it was more effective under alkaline conditions.

Effect of temperature on enzyme activity and stability

As shown in Fig. 7a, the optimal temperature for dextranase activity was found to be 40 °C, with dextranase showing activity in the range of 35 to 50 °C, with a peak relative activity of no less than 88 %. Dextranase from strain DP03 also showed 26.6 % relative activity at 0 °C. The thermal stability of crude dextranase showed that it retained almost 100 % residual activity at 30 °C (pH 7.5) for 5 h. Nearly 20 % of the activity was lost after 5 h of exposure at 40 °C (Fig. 7b).

SEM images of biofilm

The effects of dextranase on bacterial biofilms were analyzed using SEM and are presented in Fig. 8. The blank control and negative control equally showed that *S. mutans* grew well without any apparent cell lysis or cellular debris;

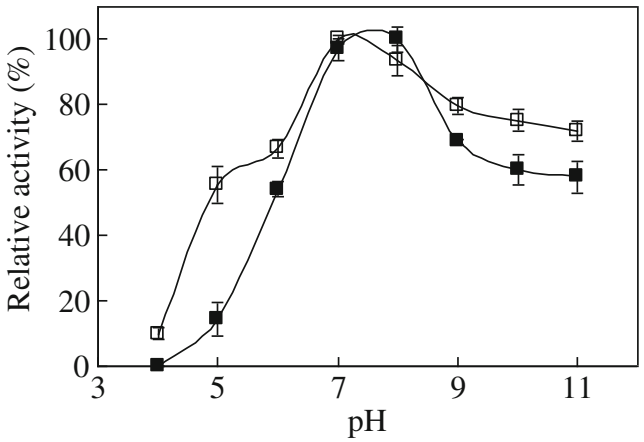

Fig. 6 Optimum pH and stability curves of *Catenovulum* dextranase. For each pH, activity was assayed at 40 °C and relative activity was assessed (*filled square*). The pH stability curve (*open square*) represents the residue activity after a preincubation period of 1 h at 25 °C. For the effect of pH on dextranase activity and stability, the values are shown as percentages of the maximum activities, which were taken to be 100 %

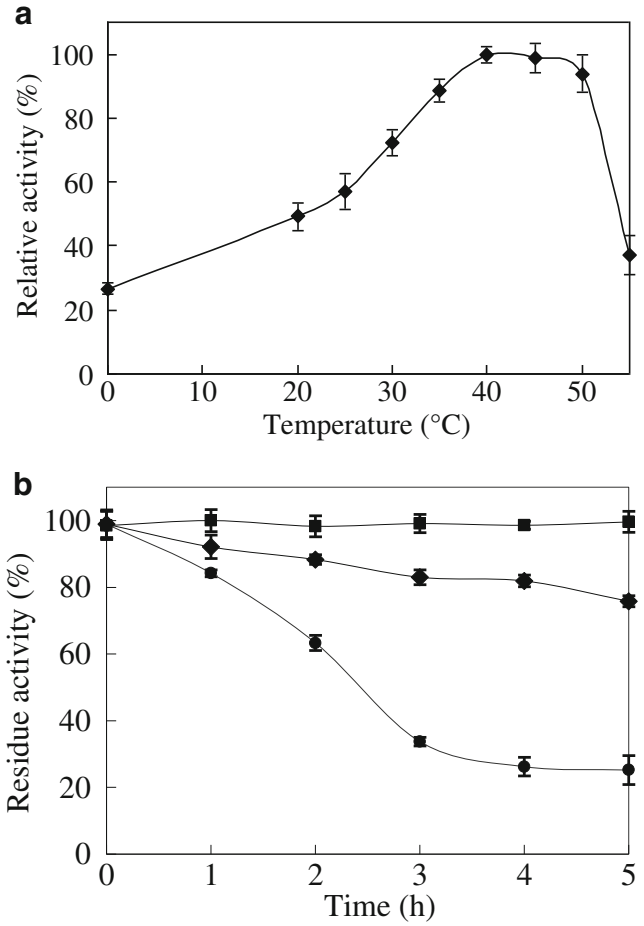

Fig. 7 Effects of temperature on activity (**a**) and thermal stability (**b**) of *Catenovulum* dextranase. Thermal stability (**b**; *filled square* 30 °C, *filled diamond* 40 °C, *filled circle* 50 °C. For the effect of temperature on the dextranase activity and stability, the values are shown as percentages of the maximum activities, which were taken to be 100 %

the biofilm displayed a bright, nearly intact surface, with no obvious breaking down of structures (Fig. 8a, b). However, we observed that the addition of 40 units of *Catenovulum* dextranase to the BHI media impeded biofilm formation and reduced the cell population of *S. mutans* adhering to the glass coverslips (Fig. 8d). A similar consequence was observed in the positive control, to which 40 units of *P. janthinellum* dextranase was added (Fig. 8c). As a result, biofilm did not form easily when *S. mutans* was co-cultured with dextranase from *Catenovulum* sp. DP03.

Discussion

Catenovulum sp. is a Gram-negative, rod-shaped, heterotrophic, aerobic, agar-hydrolyzing bacterium found in marine environments. It forms a distinct clade closely related to species of the family *Alteromonadaceae* within the group of *Alteromonas*-like gammaproteobacteria according to Yan et al. (2011). *Catenovulum* strain is a novel clade of marine bacteria that has only been identified in recent years; consequently, there is little published information on its characteristics. A 16S rRNA gene sequence of an isolate named *Catenovulum agarivorans* gen. nov. sp. nov. YM01 is the first deposition in GenBank, showing only 88.4–91.0 % sequence similarity to its closest neighbors. To date, only two *Catenovulum* 16S rRNA gene sequences have been deposited in GenBank. We isolated strain DP03 from coastal seawater. Based on morphological and biochemical characterization and 16S rRNA gene sequence comparison, we identified strain DP03 as *Catenovulum* sp. To our knowledge, this is the first report of any dextranase from *Catenovulum* sp.

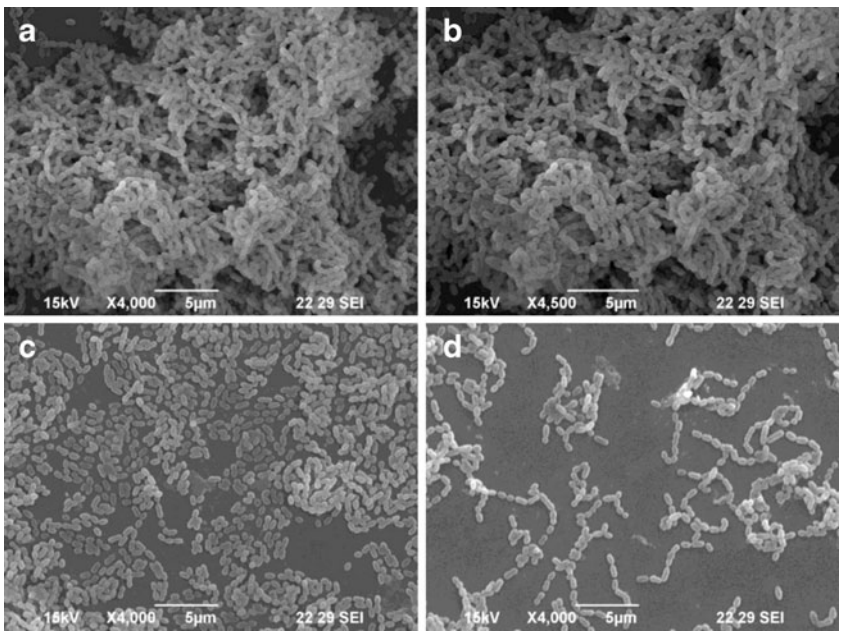

Fig. 8 Electron microscopy analysis of *Streptococcus mutans* biofilms formed on glass coverslips in the absence and presence of dextranases. **a** Blank control; note that an equal volume of cell-free pure water was added to replace dextranase. **b** Negative control; an equal volume of thermal inactivated *Catenovulum* dextranase was added. **c** Positive control; 40 units of cell-free of *Penicillium janthinellum* dextranase was added. **d** Biofilm subjected to 40 units of *Catenovulum* dextranase

Conditions promoting growth and dextranase production of *Catenovulum* sp. DP03 have been studied. Strain DP03 was found to show no growth in the absence of NaCl. It is a halophilic bacterium, like *C. agarivorans* (Yan et al. 2011). The isolate was also found to produce dextranase at lower temperatures and under alkaline conditions, similar to seawater conditions. No enzymatic activity was detected unless dextran was present in the media. Dextranase is an inducible enzyme that was first reported by Staat and Schachtele (1976). We found that strain DP03 produced more dextranase from lower-molecular-weight dextrans than from heavier ones, which is an important feature considering that the lower-molecular-weight dextrans are less costly than those of a higher molecular weight.

The optimum pH for dextranase activity was found to be 8.0. Our measured data were different from those reported in most previous studies on dextranases, which usually demonstrates optimal activities at acidic pH values (Khalikova et al. 2005). The most recently reported alkalophilic dextranase were from *Streptomyces* sp. NK458 and *Bacillus subtilis* NRC-B233b; the maximum activities of these two dextranases occurred at pH 9.0 and 9.2, respectively (Esawy et al. 2012; Purushe et al. 2012). The optimum temperature for crude dextranase action was found to be 40 °C; however, it still catalyzes the hydrolysis of dextran to oligosaccharides at 0 °C and so can be called a cold-active enzyme (Margesin et al. 2005). Crude *Catenovulum* dextranase acts efficiently within a temperature range of 35–50 °C, which is similar to the temperature of the human body and the ideal temperature for mesophilic dextran degradation.

The crude *Catenovulum* dextranase was found to be stable at a pH range of 5.0–9.0 for 1 h. As such, it is superior to the dextranases from *Sporothrix schencki* (stability range 4.5–4.75) and *Hypocrea lixii* (4–6.5) (Wu et al. 2011). Applications involving *Catenovulum* dextranase can be performed under these pH conditions to preserve enzyme activity. The thermal stability of *Catenovulum* dextranase was found to be inferior to that of dextranases from *Paecilomyces lilacinus* and an anaerobic thermophilic bacterium after 1 h of exposure (Galvez-Mariscal and Lopez-Munguia 1991; Wynter et al. 1997). However, it was equivalent to that of dextranase from *Hypocrea lixii* (Wu et al. 2011). Further study showed that the cell-free *Catenovulum* dextranase could be stored at 30 °C for 7 days and 4 °C for over 3 months without any loss of enzymatic activity (data not shown). This suggests that it can tolerate standard enzyme storage conditions.

Optimum catalytic conditions and stability studies of dextranase from *Catenovulum* sp. DP03 indicate that an alkalophilic and cold-active dextranase capable of catalysis under conditions resembling those of the human body may be suitable for used in oral rinse products and novel treatments for oral dental plaques. We performed a very preliminary study to verify that dextranase from *Catenovulum* sp. DP03 can prevent the formation of biofilms. *S. mutans* was selected as a model organism for the construction of a biofilm, similar to those which adhere to human teeth. The presence of sucrose can favor the formation of extracellular polysaccharides (glucans) which, in turn, seems to enhance the adhesion of *S. mutans* to surfaces (Schilling and Bowen 1992; Rahim and Thurairajah 2011). As shown in the SEM study, dextranase produced by *Catenovulum* sp. DP03 was able to hinder biofilm formation in the *S. mutans* model. The positive control study performed using a purified *P. janthinellum* dextranase showed a similar result. Also, the results from negative controls indicate that the *Catenovulum* dextranase was the agent preventing extensive biofilm formation by *S. mutans*. The reduced biofilm is attributable to the failure of extracellular polysaccharide to form efficiently, which is due to cleavage of the α-1,6 glucosidic linkages of the biofilm that occurs in the presence of dextranase. Previous studies have used an SEM technique that facilitates determination of the relation between medical agents (including but not limited to dextranase) and *S. mutans* biofilm (Ota and Fukui 1982; Rahim and Thurairajah 2011; Tao et al. 2011). More studies of the *Catenovulum* dextranase in the treatment of dental caries are ongoing, although existing data show its potential industrial and medical value.

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