TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2013) 37: 520-529
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doi:10.3906/biy-1206-3

Production and characterization of uricase from *Streptomyces exfoliatus* UR10 isolated from farm wastes

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Received: 03.06.2012 • **Accepted:** 04.01.2013 • **Published Online:** 06.09.2013 • **Printed:** 04.10.2013

Abstract: Uricase plays an important role in nitrogen metabolism and can be used medically as a diagnostic reagent. From soil, wastewater, and poultry waste samples collected in Jeddah, 49 bacterial isolates were obtained on either nutrient agar or starch nitrate agar. All the obtained isolates were screened on minimal medium containing 0.5% uric acid for uricase production. The most active bacterium (isolate UR10) produced about 0.5 U/mL of intracellular uricase and was identified as a species belonging to the genus *Streptomyces* using morphological, physiological, and biochemical characters. By 16S rDNA, it was identified as *Streptomyces exfoliatus* UR10. Maximum uricase production was obtained using medium 2 with 0.2% uric acid as an inducer, an initial pH of 6.5, and an incubation temperature of 37 °C at 100 rpm. At the end of the incubation period, the cells were collected and disturbed, and the uricase enzyme was precipitated by ammonium sulfate. The enzyme was purified using different column chromatography methods, and the molecular weight of the purified uricase was determined by SDS-PAGE electrophoresis. The optimum temperature for maximum uricase activity was 45 °C; the optimum pH was 8. Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and Pb^{2+} decreased the enzyme activity, whereas Ca^{2+}, Mn^{2+}, Mg^{2+}, and Fe^{2+} stimulated it. In conclusion, uricase was produced by *Streptomyces* in a medium containing uric acid as inducer, and this enzyme can be used to detect and quantify uric acid in urine and/or blood.

Key words: Uricase, *Streptomyces*, molecular weight, uric acid, enzyme activity, 16S rDNA

1. Introduction

The use of intracellular enzymes for analytical and medical purposes is becoming increasingly popular. Uricase (urate oxidase) is an enzyme that participates in the purine breakdown pathway, catalyzing the oxidation of uric acid to allantoin and hydrogen peroxide in the presence of oxygen. Some species of animals and birds have lost the uricase gene and are therefore unable to degrade urate (1). The absence of uricase in humans leads to gout, which is caused by an accumulation of uric acid. Pegloticase (commercial uricase) is a recombinant uricase that can be used in humans to lower the levels of uric acid by catalyzing the oxidation of uric acid to allantoin, which is then eliminated via the kidneys. Thus, uricase is a promising enzyme with a high specificity toward uric acid. It is usually needed in large quantities for medical uses including the analysis of human serum or urine for uric acid and as a protein drug to reduce toxic urate accumulation (2).

Uricase is mainly localized in the liver of animals and inside microorganisms, especially bacteria such as *Bacillus pasteurii* (3), *Proteus mirabilis* (4), and *Escherichia coli* (5). Ammar et al. (6) stated that *Streptomyces albosriseolus* potentially produced uricase in media that contained uric acid as the main source of carbon, nitrogen, and energy. A thermostable uricase was obtained from *Microbacterium* (7) and *Bacillus thermocatenulatus* (8). Tanaka et al. (9) demonstrated that in addition to uric acid, xanthine, guanine, adenine, and hypoxanthine were also effective for inducing uricase in *Candida tropicalis*. Abdel-Fattah et al. (10) found that glucose, the medium used, pH, $CuSO_4$, and $FeSO_4$ all had a highly significant effect on the uricase activity produced by *Pseudomonas aeruginosa*, based on statistical experimental designs. The aim of this study was the isolation and characterization of a uricase-producer bacterium and optimization of growth conditions for maximum enzyme production. Moreover, the obtained uricase was purified and characterized.

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2. Materials and methods

2.1. Sample collection

Samples (soil, wastewater, marine water, and poultry wastes) were collected from different places in Jeddah, Saudi Arabia. All samples were transported in sterile plastic bags to the laboratory, and all samples, except the water, were spread on paper sheets to air-dry.

2.2. Isolation and purification of the bacterial isolates

Soil and farm waste samples were ground in a mortar and sieved using a 4-mm mesh screen. From each sieved sample, 1 g was stirred into 100 mL of sterile distilled water for 5 min in a 250-mL Erlenmeyer flask, and the suspension was allowed to stand for 30 min. Serial dilutions from each sample were prepared. From the appropriate dilution or collected water sample, 1 mL was spread on both nutrient agar and starch nitrate agar (11) media. The plates were incubated at 30 °C for 2–7 days, and the developed colonies were purified and preserved using nutrient agar.

2.3. Screening of all bacterial isolates for uricase production

All the isolated and purified bacteria were screened for uricase activity using the plate method, with a medium containing 0.1% glucose, 0.1% yeast extract, 0.5% uric acid, and 2% Oxoid agar (12).

2.4. Cultivation of bacteria-producing uricase in liquid media

A 2-stage submerged cultivation was carried out in 500-mL Erlenmeyer flasks containing 100 mL of growth medium (2% peptone, 3% glucose, 0.1% K_2HPO_4, 0.05% NaCl, and 0.01% uric acid) in a shaker (120 rpm) at 30 °C for 2 days. About 5 mL (5×10^5 CFU/mL) was transferred to 95 mL of production medium, consisting of 5% sucrose, 3% corn steep liquor, 0.1% uric acid, 0.1% proteose-peptone, 0.05% biotin, 0.1% KCl, and 0.1% NaCl at pH 6.2 (13). After 36 h samples were taken for determination of bacterial growth ($A_{550\ nm}$) and uricase activity in both filtrate and cells (14).

2.5. Enzyme assay

Cells were harvested by centrifugation (5000 rpm, 15 min) and disintegrated with an equal amount of glass beads at 4 °C for 10 min. Uricase activity was measured according to the procedure described by Adamek et al. (14). The calibration curve was prepared from commercial uricase with an enzyme activity of 5 U/mL.

2.6. Characterization of the selected actinomycete isolate

The actinomycete selected as the best uricase-producing organism was characterized and identified. Morphological studies were conducted after growth on oatmeal agar medium using light and electron microscopy. Some physiological characters, carbon and nitrogen utilization, and sensitivity of the selected bacterium on Muller–Hinton agar to different antibiotics were carried out as described by Aly et al. (15). Analysis of the isomer of diaminopimelic acid and whole-cell sugar composition were studied following the procedure described by Hasegawa et al. (16). Fatty acid methyl esters were prepared (17) and determined using gas chromatography. Phospholipid types were determined by 2-dimensional thin-layer chromatography (18).

2.7. Phylogenetic analysis of 16S rDNA sequence

Genomic DNA from the selected isolate was obtained using the QIAamp DNA Mini Kit (19). The 16S rDNA gene was amplified by PCR using the forward primer 5'-AGTTTGATCATGGTCAG-3' and the reverse primer 5'-GGTTACCTTGTTACGACT-3'. The DNA sequence was compared to the GenBank database at the National Center for Biotechnology Information (NCBI) using the BLAST program.

2.8. Optimization of the uricase production process

The effects of different factors on intracellular uricase production by the selected bacterium UR10 were determined. Optimization studies were selected based on maximum uricase production. Different uric acid concentrations were evaluated as inducers (20), and the effects of 6 different media with varying compositions on uricase production were also studied. The media used were M1 (21), M2 (22), M3 (20), M4 (20), M5 (23), and M6 (20). These were then compared with the control, M7 (13). The effects of different temperatures (20, 25, 30, 35, 37, and 40 °C), pH values (6.0, 6.5, 7.0, 7.5, and 8.0), incubation periods (1 to 7 days), and shaking rates were determined, as described by Aly et al. (24,25). After incubation the growth and uricase production of the selected bacterium were determined.

2.9. Purification of uricase and molecular weight determination

After growth, the cells were collected, washed, and broken down, and cell proteins were precipitated with 80% ammonium sulfate. The precipitate was dialyzed, concentrated under vacuum, and applied to a column of DEAE cellulose followed by carboxymethyl-cellulose and Sephadex G-75. Elution was carried out with 1 M NaCl in phosphate buffer. The molecular weight of the purified uricase was determined from a standard protein marker (24).

2.10. Properties of the purified uricase

The purified enzyme mixture was incubated at different temperatures (30, 35, 40, 45, 50, 55, and 60 °C) for different amounts of time (30–60 min), and uricase activity was detected. Uricase activity was studied at 45 °C at different pH values, substrates, and enzyme concentrations and in the presence of metal ions or chemicals (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Li^{+}, Ag^{+}, Co^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, $PO4^{3-}$, and EDTA) at 10^{-3} M. At the end of the incubation period (30 min), tubes were cooled and uricase was assayed as previously mentioned.

2.11. Statistical analysis

The means of variable and standard deviations were recorded. Data were subjected to statistical analysis using SPSS 16, and the differences between mean values as determined by Student's t-test were considered significant at $P < 0.05$.

3. Results

The bacterial isolates used in this study were recovered from contaminated soils, marine water, poultry farm wastes, and wastewater samples collected from different localities in Jeddah, Saudi Arabia. Forty-nine bacterial isolates were obtained on either nutrient agar or starch nitrate agar. All the isolates were screened on agar medium supplemented with uric acid. After 2–7 days of cultivation at 30 °C, 10 isolates (20%) produced uricase, which was detected as a clear zone around the bacterial colonies. All uricase-producing bacteria were grown in liquid medium containing uric acid as a nitrogen source, and quantities of uricase were determined in both the cultural supernatant as well as the intracellular fluid. It was found that the intracellular uricase ranged from 0.09 to 0.5 U/mL, whereas the extracellular ranged from 0.08 to 0.43 U/mL. The most active intracellular uricase producer was the isolate UR10 (Table 1), obtained from poultry farm waste. The isolates UR3 and UR5 grew well using uric acid as carbon and nitrogen sources; however, they were weak producers of uricase.

Examination of isolate UR10 under a light microscope revealed that it was a gram-positive spore-chain–forming bacterium with a filamentous structure including aerial and substrate mycelia (Figure 1). Isolate UR10 was grown on different growth media, and the aerial substrate mycelia were described in addition to the soluble pigment production. The growth ranged from heavy and moderate to poor (Table 2). The selected bacterial isolate was characterized by morphological, physiological, and biochemical properties (Tables 3 and 4). Analyses of cell wall and whole cell hydrolysates revealed the L-isomer of diaminopimelic acid and glucose in addition to phosphatidylethanolamine and many branched and unbranched saturated fatty acids (Table 5). The results of 16S rDNA showed 97% identity with the homologous fragments of *Streptomyces exfoliatus* (Figure 2).

Growth and uricase production varied by inducer added, medium used, incubation period, incubation temperature, initial pH, and shaking rate. The results showed that 0.2% uric acid was the most effective inducer. Uricase induction started after 4 h, rapidly increased during the first 12 h of cultivation, slowly increased until it reached the maximum level (0.5 U/mL) after 72 h, and then decreased to 0.3 U/mL after 96 h (Figure 3). For uricase production by the selected bacterium, 6 different media containing uric acid as the inducer were used, and uricase was measured at the end of the growth period (3 days). The best medium for uricase production was M2, followed by M7 (Figure 4). The most suitable temperature was 37 °C, and increasing the temperature above the optimum level decreased uricase production (Figure 5). The effects of initial medium pH on uricase production are shown in Figure 6. Maximum production was obtained at an initial pH of 6.5 and production dropped significantly at pH 9.0. The highest level of uricase was found after 3 days of growth at 100 rpm (Figures 7 and 8).

Streptomyces exfoliatus UR10 was grown using the optimal conditions for uricase production: in M2 with

Table 1. Growth and uricase production by different bacterial isolates obtained from different sources collected from Jeddah.

Isolate no.	Source	Gram reaction	Uricase detection on solid medium	Liquid medium		
				Growth (cfu/mL × 10^4)	Uricase production	
					Inside the cells	Outside the cells
US1	Soil	Negative	+	1.3	0.40	0.32
UR 2	Soil	Negative	+	1.0	0.30	0.43
UR 3	Soil	Negative	++	4.0	0.08	0.16
UR 4	Farm wastes	Negative	++	1.0	0.30	0.09
UR 5	Marine water	Negative	++	5.5	0.18	0.22
			Isolated actinomycetes			
UR 6	Soil	Positive	++	0.01	0.07	0.33
UR7	Soil	Positive	++	0.9	0.19	0.23
UR8	Soil	Positive	++	0.7	0.89	0.09
UR9	Farm wastes	Positive	++	1.0	0.09	0.11
UR10	Farm wastes	Positive	++	1.3	0.50	0.08

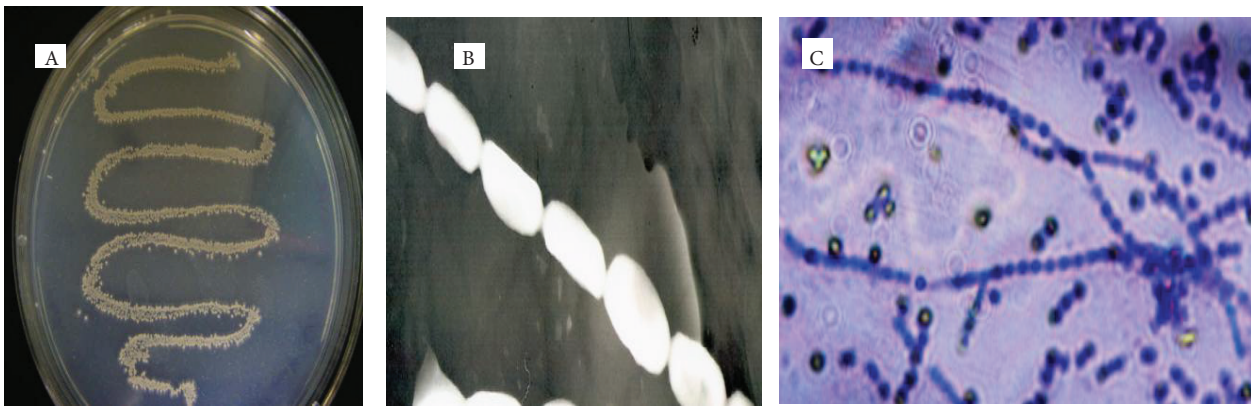

Figure 1. The isolated bacterium grown on uric acid medium (A), under electron microscope at 1,5000× (B), and under light microscope 1000× (C).

Table 2. Cultural characteristics of the actinomycete isolate UR10 grown on different agar media at 30 °C.

Media	Growth	Color of aerial mycelium	Color of substrate mycelium	Presence of soluble pigment
Starch nitrate agar	Heavy	Pink	Brown	-
Glucose asparagine agar	Heavy	Pale yellow	Yellow	-
Inorganic salts starch iron agar (ISP-4)	Moderate	Pale yellow	Yellowish white	-
Tyrosine agar (ISP-7)	Moderate	Yellow	Pale yellow	-
Yeast extract malt extract agar (ISP-2)	Moderate	Yellowish brown	Pink	-
Oatmeal agar (ISP-3)	Poor	Yellow	Pale yellow	-
Glycerol asparagine agar (ISP-5)	Moderate	Dark yellow	Yellow	-

-: no melanin pigment detected.

Table 3. Morphological characters of the tested isolate, UR10.

Tested character	Result
Gram stain	Gram-positive
Motility of spore	Absent
Shape of spore	Cylindrical (5–6 and 6–9 μm)
Spore chain	Straight chain
Spore surface	Smooth
Number of spores/chains	14–24
Aerial and substrate mycelia	Well developed
Zoospore, sporangium, sclerotia, and fragmented mycelia	Absent

initial pH 6.5 and incubation temperature of 37 °C at 100 rpm for 3 days. The enzyme was extracted from the cell, purified, and characterized. Uricase purification was carried out using column chromatography (Figure 9). The molecular weight of the purified uricase was 43 KDa, as detected by gel electrophoresis (Figure 10). The purified enzyme was stable at 45 °C for 60 min; increasing the temperature up to 50 °C reduced the activity to 90%, 80%, and 70% after 30, 45, and 60 min, respectively (Figure 11). At 60 °C, the activity decreased to 10% after 60 min. The activity of the enzyme was measured in different buffers ranging from pH 4.0 to pH 11.0. After incubation in different buffers at 37 °C for 30 min, maximum uricase activity was obtained at pH 8 (Figure 12).

Table 4. Physiological characteristics of isolate UR10.

Character	Result	Utilization of carbon and nitrogen sources	Result
Melanin pigment on tyrosine agar	+ve	Carbon source	
Proteolysis	+ve	Glucose	++
Lecithinase	-ve	D-mannitol	++
Lipolysis	+ve	Glycerol	++
Chitinase	+ve	Raffinose	--
Gelatinase	+ve	D-galactose	--
Pectinase	-ve	Sucrose	++
H2S production	-ve	Starch	++
Growth temperature	15–45 °C	D-xylose	--
Tolerance to NaCl	5%–12%	Nitrogen sources	
pH range	6–9	KNO_3	++
Resistance to antibiotics		Valine	++
Penicillin	+	Phenylalanine	++
Cephalosporin	+	NH_4Cl	++
Kanamycin	+	$Na\ NO_3$	++
Rifampin	-	$NaNO_2$	--

-ve: Negative result, +ve: positive result, +: resistance, -: sensitive, ++ utilization, --: no utilization.

Table 5. Sugar, amino acid, phospholipid, and fatty acid composition of the cell wall or cell hydrolysate of the tested isolate, UR10.

Type of reaction	Results
- Diagnostic sugar	
Glucose	+
- Amino acids	
Diaminopimelic acid	+ (L-form)
Glutamic acid	+
Glycine	+
Alanine	-
Lysine	-
-Phospholipids	
Phosphatidylethanolamine	+
Phosphatidylinositol	-
-Fatty Acids	
Iso, anteiso fatty acid	+

+: present. -: absent.

The effect of enzyme concentration on uricase activity was detected, and it was found that increasing enzyme concentration increased enzyme activity (Figure 13). Furthermore, increasing uric acid concentration of up to 5 mg/mL enhanced uricase activity; however, higher concentrations had no significant effect on uricase activity (Figure 14). Among the metal ions, Li^+, Ag^+, and Hg^+ significantly inhibited enzyme activity whereas Ca^{+2} and Fe^{+2} significantly enhanced uricase activity (Figure 15). When EDTA, a chelating reagent, was added to the enzyme solution at a final concentration of 20 mM, uricase activity was slightly inhibited.

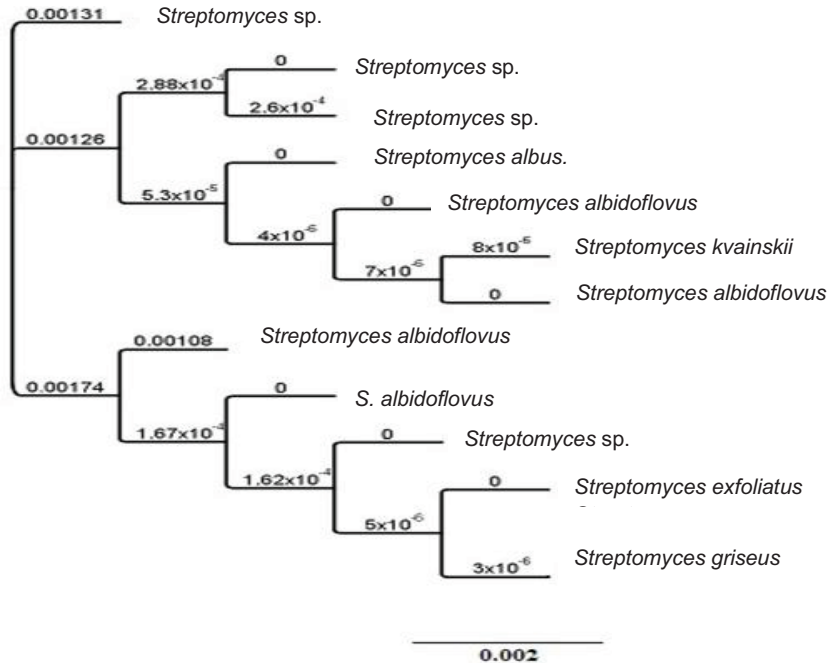

Figure 2. Phylogenetic tree based on 16S rDNA sequence comparisons of *Streptomyces* UR10 by neighbor joining tree method. Maximum sequence difference = 0.002.

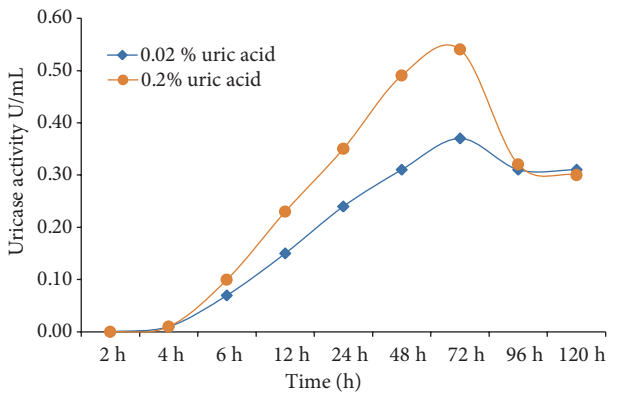

Figure 3. Effect of different concentrations of uric acid on uricase induction (U/mL) by the selected isolate, UR10.

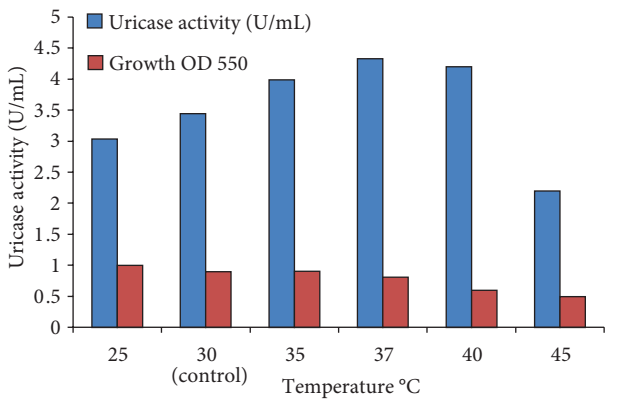

Figure 5. Effect of different temperatures on growth and uricase production by the selected isolate, UR10.

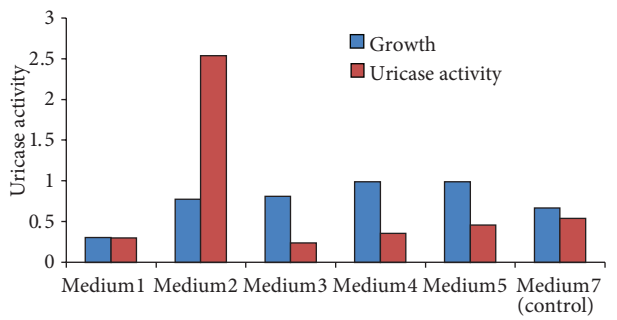

Figure 4. Effect of different media on growth and uricase production by the selected isolate, UR10.

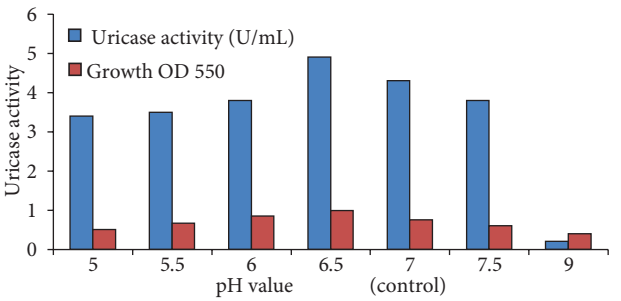

Figure 6. Effect of different pH levels on growth and uricase production by the selected isolate, UR10.

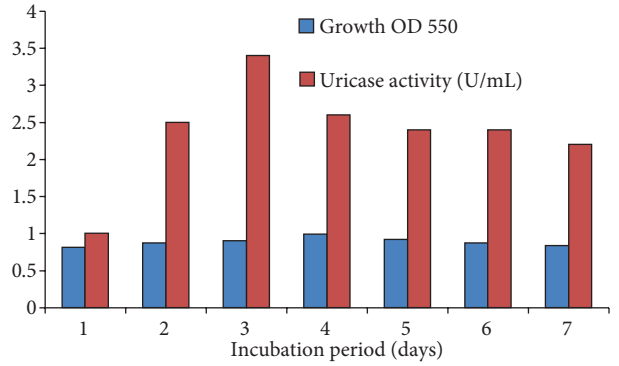

Figure 7. Effect of different incubation periods on growth and uricase production by the selected isolate, UR10.

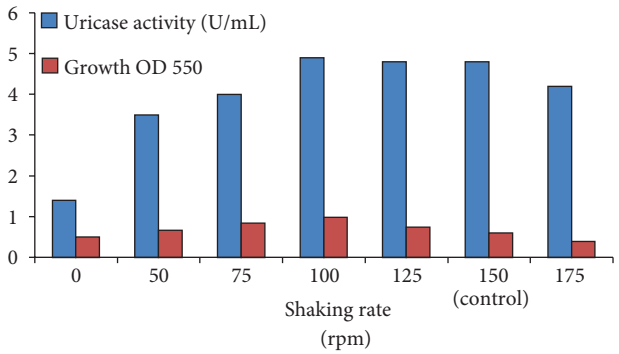

Figure 8. Effect of different shaking rates on growth and uricase production by the selected isolate, UR10.

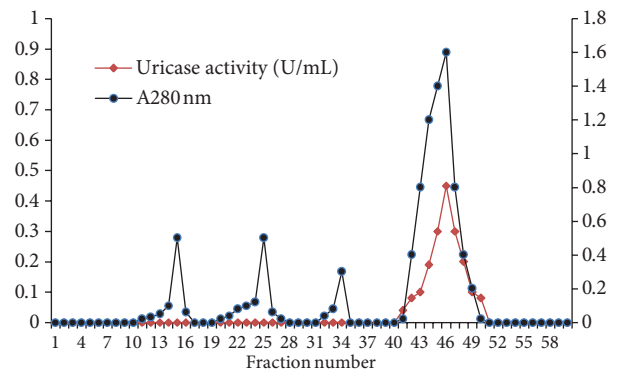

Figure 9. Profile of elution of uricase using DEAE cellulose column chromatography.

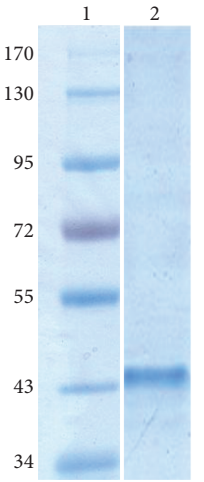

Figure 10. SDS-PAGE profile of purified uricase. Lane 1: standard protein marker, lane 2: purified uricase.

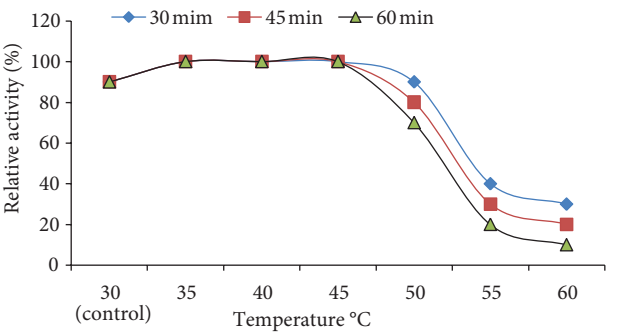

Figure 11. Effect of different temperatures on uricase activity after 30, 45, and 60 min.

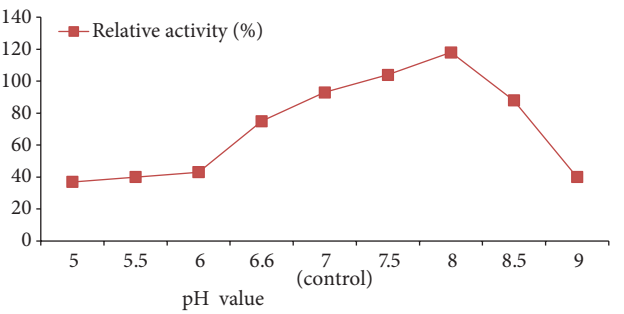

Figure 12. Effect of different pH values on relative uricase activity.

4. Discussion

Uricase plays an important general role in nitrogen metabolism and can be used medically as a diagnostic reagent. Uricase was isolated from mammalian organisms, but more recently it has been obtained from various fungi and bacteria. In all cases, the enzyme was found to be inducible, and the presence of uric acid or some other inducer in the medium is necessary for enzyme formation (26). In this work, 20% of the screened bacteria were uricase-producing. Lookwood and Garrison (27) reported that several microorganisms could utilize uric acid as their sole source of nitrogen or satisfy their nitrogen and carbon requirements through the production of uricase; this activity was detected as clear zones accompanying the growth of microorganisms in solid agar (28). In isolate UR10, uricase production was intracellular, and

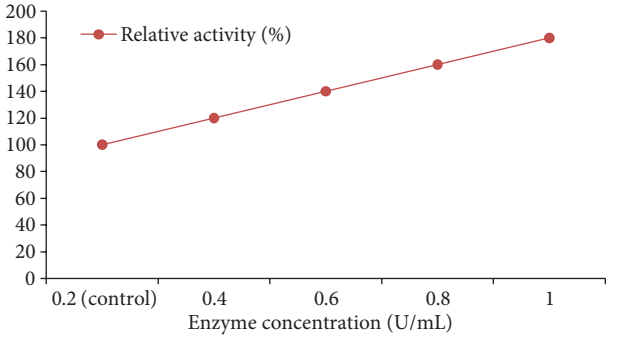

Figure 13. Effect of different enzyme concentrations on uricase activity.

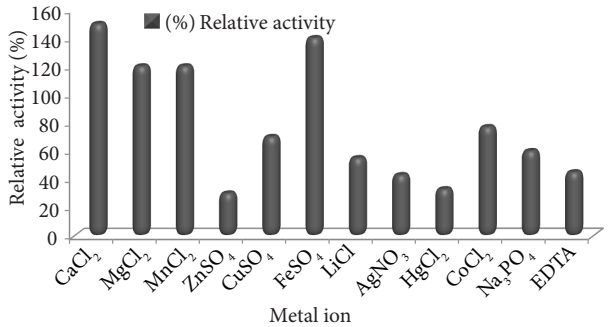

Figure 15. Effect of different metal ions and EDTA on uricase activity.

a little uricase activity was found in the supernatant. Moreover, there is no significant relationship between bacterial growth and uricase production. Similarly, Nour El-Dein and El-Fallal (29) speculated that uricase production is not a function of fungal or bacterial growth. Khucharoenphaisan and Sinma (30) obtained similar results in *Saccharopolyspora* sp. and Azab et al. (26) in 2 strains of *Proteus* and 3 species of *Streptomyces*. Biochemical analysis of the cell wall of isolate UR10 indicated a wall of chemotype IV; the whole-cell sugar pattern was type A. The phospholipid pattern was type PII, and the fatty acids detected using gas chromatography were pattern c. According to morphological, physiological, and biochemical comparison analysis of the characteristics of isolate UR10 and other described isolates (31), UR10 belongs to the genus *Streptomyces*. The identification results were confirmed using 16S rDNA, which is considered a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria, and eukaryotic organisms (32). The 16S rDNA sequence was compared to the GenBank database at the NCBI using the BLAST program. According to the obtained results, UR10 was identified as *Streptomyces exfoliatus* UR10, which is a new uricase producer. Similarly, in India, 4 strains belonging to *Streptomyces exfoliatus* were isolated from mangrove ecosystems (33). *Streptomyces*

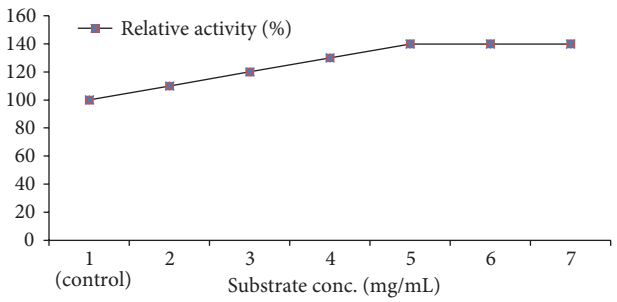

Figure 14. Effect of different concentrations of uric acid on uricase activity.

graminofaciens and *Streptomyces albidoflavus* were intracellular uricase producers in medium containing uric acid (26). Maximum enzyme production occurred after 72 h using 2% uric acid as an inducer, which was higher than in *Microbacterium* ZZJ4-1 (12 h) (7). Our results were in agreement with those of Demnerova et al. (20) and Azab et al. (26). Moreover, media containing uric acid supported uricase production, which was in accordance with the findings of Atalla et al. (34). In contrast to our results, incubation at 30 °C and 150 rpm for 8 days (34) or 24 h (35) was required for maximum enzyme production. The optimum pH for maximum uricase production was 6.5, which is somewhat in agreement with the results of Yazdi et al. (36) and Tohamy and Shindia (37). For maximum enzyme production, higher pH values (8.8, 9.2, and 10) were recorded by many authors (37–39). Increasing the shaking rate enhanced aeration level, which favors uricase production (32). *Streptomyces exfoliatus* UR10 was considered an excellent intracellular uricase producer. Most of the microbial uricase from *Microbacterium* ZZJ4-1, *Proteus vulgaris* 1753, *P. vulgaris* B317-C, *Streptomyces graminofaciens*, *Streptomyces albidoflavus*, and *Streptomyces cyanogenus* (7,26,38,39) was intracellular, and cell disruption was necessary to obtain the enzyme. However, in some microbial resources such as *Bacillus fastidiosus* (40) and *Pseudomonas aeruginosa* (41), extracellular uricase has been found without cell disruption. The purified enzyme showed a single protein band in SDS-PAGE with 44 kDa. Uricases from different sources may have different molecular masses and amino acid sequences. In another study, the molecular mass of the uricase was estimated at 34–54 kDa (42).

The purified enzyme is a thermotolerant uricase and can be used in many clinical applications where thermostability is an important characteristic (43). For maximum uricase activity, the optimum temperature and pH were 45 °C and pH 8.5 (44). Some uricases require certain metal ions or cofactors that are strongly bonded and form part of the uricase structure. These ions are very

important for maintaining maximum catalytic activity (45). Li^{+}, Ag^{+}, and Hg^{+} ions and the chelating reagent (20 mM EDTA) greatly inhibited the enzyme activity. In some cases, uricase can exist as a tetramer of identical subunits, each containing 2 copper-binding sites (45); this property was different in uricase from *Arthrobacter globiformis* (46). Furthermore, Cu^{2+}, Fe^{3+}, Ag^{+}, and $Zn2^{+}$ were strong uricase inhibitors; however, they did not inhibit *Arthrobacter* uricase. In conclusion, pure uricase can be produced from bacteria and can be used to lower uric acid levels and assay uric acid in blood or urine.

Acknowledgments
This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, Saudi Arabia (grant no. 1431/247/353). The authors acknowledge the technical and financial support of the DSR with gratitude.

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