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Production and Characterization of Urease from *Streptomyces chromofuscus* Isolated from the Soil at Saudi Arabia

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From soil, collected in Riyadh, 49 bacterial isolates were obtained on starch nitrate agar. All the obtained isolates were screened on minimal medium containing 0.5 % uric acid for urease production. The most active bacterium (isolate UR10) produced about 0.5 U/mL of intracellular urease 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 chromofuscus* UR10. Maximum urease 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 urease enzyme was precipitated by ammonium sulfate. The enzyme was purified using different column chromatography methods, and the molecular weight of the purified urease was determined by SDS-PAGE electrophoresis. The optimum temperature for maximum urease 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, urease 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: Urease, *Streptomyces*, molecular weight, uric acid, enzyme activity, 16S rDNA.

The use of intracellular enzymes for analytical and medical purposes is becoming increasingly popular. Urease (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 urease gene and are therefore unable to degrade urate[1]. The absence of urease in humans leads to gout, which is caused by an accumulation of uric acid. Pegloticase (commercial urease) is a recombinant urease 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, urease 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]. Urease 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 urease in media that contained uric acid as the main source of carbon, nitrogen, and energy. A thermostable urease 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 urease 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 urease activity produced by *Pseudomonas aeruginosa*, based on statistical

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experimental designs. The aim of this study was the isolation and characterization of a urease-producer bacterium and optimization of growth conditions for maximum enzyme production. Moreover, the obtained urease was purified and characterized.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from different places in Riyadh, Saudi Arabia. All samples were transported in sterile plastic bags to the laboratory, and all samples were spread on paper sheets to air-dry.

Isolation and purification of the bacterial isolates

Soil samples were ground in a mortar and sieved using a 4-mm mesh screen. From each sieved sample, 1 gm 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, 1 mL was spread on starch nitrate agar[11] medium. The plates were incubated at 30°C for 2–7 days, and the developed colonies were purified and preserved using starch nitrate agar.

Screening of all bacterial isolates for urease production

All the isolated and purified bacteria were screened for urease 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].

Cultivation of bacteria-producing urease 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 × 105 CFU/mL) was transferred to 95 mL of production medium, consisting of 5 % sucrose, 3 % corn steep liquor, 0.1 % uric acid, 0.1 % protease-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 (A550 nm) and urease activity in both filtrate and cells[14].

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. Urease activity was measured according to the procedure described by Adamek *et al*.[14]. The calibration curve was prepared from commercial urease with an enzyme activity of 5 U/mL.

Characterization of the selected actinomycete isolate

The actinomycete selected as the best urease-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 was 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, 19].

Optimization of the urease production process

The effects of different factors on intracellular urease production by the selected bacterium UR10 were determined. Optimization studies were selected based on maximum urease production. Different uric acid concentrations were evaluated as inducers[20], and the effects of 6 different media with varying compositions on urease production were also studied. The media used were M1[21], M2[22], M3[23], M4[24], 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 urease production of the selected bacterium were determined.

Purification of urease 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 1M NaCl in

phosphate buffer. The molecular weight of the purified urease was determined from a standard protein marker[24].

Properties of the purified urease

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 urease activity was detected. Urease 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 urease was assayed as previously mentioned.

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 $P < 0.05$.

RESULTS

The bacterial isolates used in this study were recovered from contaminated soil samples collected from different localities in Riyadh, Saudi Arabia. Forty-nine bacterial isolates were obtained on 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 urease, which was detected as a clear zone around the bacterial colonies. All urease-producing bacteria were grown in liquid medium containing uric acid as a nitrogen source, and quantities of urease were determined in both the cultural supernatant as well as the intracellular fluid. It was found that the intracellular urease 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 urease producer was the isolate UR10 (Table 1). The isolates UR3 and UR5 grew well using uric acid as carbon and nitrogen sources; however, they were weak producers of urease. 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 hydrolysate revealed the L-isomer of diaminopimelic acid and glucose in addition to phosphatidyl-ethanolamine and many branched and un-branched saturated fatty acids (Table 5). The results of 16S rDNA showed 97 % identity with the homologous fragments of *Streptomyces chromofuscus*.

Growth and urease 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. Urease 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. For urease production by the selected bacterium, 6 different media containing uric acid as the inducer were used, and urease was measured at the end of the growth period (3 days). The best medium for urease production was M2, followed by M7. The most suitable temperature was 37°C, and increasing the temperature above the optimum level decreased urease production. The effects of initial medium pH on urease production are shown in. Maximum production was obtained at an initial pH of 6.5 and production dropped significantly at pH 9.0. The highest level of urease was found after 3 days of growth at 100 rpm. *Streptomyces chromofuscus* UR10 was grown using the optimal conditions for urease production: in M2 with 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. Urease purification was carried out using column chromatography. The molecular weight of the purified urease was 43 KDa, as detected by gel electrophoresis (Fig. 2). 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. 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 urease activity was obtained at pH 8. The effect of enzyme concentration on urease activity was detected, and it was found that increasing enzyme concentration increased enzyme activity. Furthermore, increasing uric acid concentration of up to 5 mg/mL enhanced urease activity; however, higher concentrations had no significant effect on urease activity. Among the metal ions, Li^{+}, Ag^{+}, and Hg^{+} significantly inhibited enzyme activity whereas Ca^{+2} and Fe^{+2} significantly enhanced urease activity. When EDTA, a chelating reagent, was added to the enzyme solution at a final concentration of 20 mM, urease activity was slightly inhibited.

Table 1. Growth and urease production by different bacterial isolates obtained from different soils from Riyadh

Isolate No.	Urease detection on solid medium	Liquid medium		
		Growth	Urease production	
		(cfu/mL × 10^4)	Inside the cells	Outside the cells
UR1	+	1.3	0.40	0.32
UR2	+	1.0	0.30	0.43
UR3	++	4.0	0.08	0.16
UR4	++	1.0	0.30	0.09
UR5	++	5.5	0.18	0.22
UR6	++	0.01	0.07	0.33
UR7	++	0.90	0.19	0.23
UR8	++	0.7	0.89	0.09
UR9	++	1.0	0.09	0.11
UR10	+++	1.3	0.50	0.08

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	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	-

Table 3. Morphological characteristics of isolate UR10

Test	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

DISCUSSION

Urease plays an important general role in nitrogen metabolism and can be used medically as a diagnostic reagent. Urease 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 urease-producing. Look wood 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 urease; this activity was detected as clear zones accompanying the growth of microorganisms in solid agar[28]. In isolate UR10, urease production was intracellular, and a little urease activity was found in the supernatant. Moreover, there is no significant relationship between bacterial growth and urease production. Similarly, Nour El-Dein and El-Fallal[29] speculated that urease 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*.

Table 4. Physiological characteristics of isolate UR10

Character	Result	Character	Result
Melanin pigment	+ve	Carbon sources	
Proteolysis	+ve	Glucose	++
Lecithinase	-ve	D-mannitol	++
Lipolysis	+ve	Glycerol	++
Chitinase	+ve	Raffinose	—
Gelatinase	+ve	D-galactose	—
Pectinase	-ve	Sucrose	++
H_2S production	-ve	Starch	++
Growth temperature	15–45°C	D-xylose	—
Tolerance to NaCl	5%–12 %	Nitrogen sources	
pH range	6–9	KNO_3	++
Antibiotics resistance		Valine	++
Penicillin	+	Phenylalanine	++
Cephalosporin	+	NH_4Cl	++
Kanamycin	+	$NaNO_3$	++
Rifampin	-	$NaNO_2$	—

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

Table 5. Cell wall hydrolysate of isolate, UR10

Type of reaction	Result
Glucose	+
Diaminopimelic acid	LL-form
Glutamic acid	+
Glycine	+
Alanine	-
Lysine	-
Phosphatidylethanolamine	+
Phosphatidylinositol	-
Iso, anteiso fatty acid	+

Fig. 1. The isolate UR10 grown on (A) uric acid medium, (B) under electron microscope at 1,5000×, and under light microscope 1000× (C)

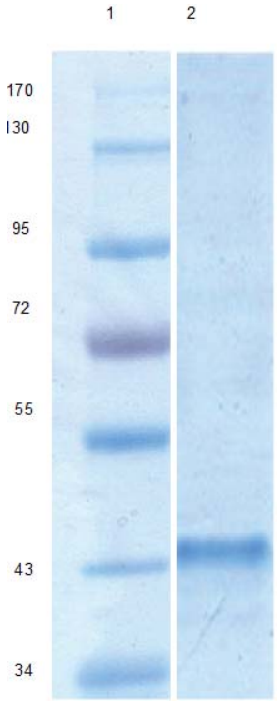

Fig. 2. SDS-PAGE profile of purified urease. Lane 1: standard protein marker, lane 2: purified urease

Biochemical analysis of the cell wall of isolate UR10 indicated a wall of chemo-type IV; the whole-cell sugar pattern was type A. The phospholipid pattern was type PII, and the fatty acids detected using gas chromatography was 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 chromofuscus* UR10, which is a new urease producer. Similarly, in India, 4 strains belonging to *Streptomyces chromofuscus* were isolated from mangrove ecosystems[33]. *Streptomyces graminofaciens* and *Streptomyces albidoflavus* were intracellular urease 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 urease 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 urease 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[37,38,39]. Increasing the shaking rate enhanced aeration level, which favors urease production[32]. *Streptomyces chromofuscus* UR10 was considered an excellent intracellular urease producer. Most of the microbial urease 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 urease has been found without cell disruption.

The purified enzyme showed a single protein band in SDS-PAGE with 44 KDa. Ureases from different sources may have different molecular masses and amino acid sequences. In another study, the molecular mass of the urease was estimated at 34–54 KDa[42]. The purified enzyme is a thermo-tolerant urease and can be used in many clinical applications where thermostability is an important characteristic[43]. For maximum urease activity, the optimum temperature and pH were 45°C and pH 8.5[44]. Some ureases require certain metal ions or cofactors that are strongly bonded and form part of the urease 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, urease can exist as a tetramer of identical subunits, each containing 2 copper-binding sites[45]; this property was different in urease from *Arthrobacter globiformis*[46]. Furthermore, Cu^{2+}, Fe^{3+}, Ag^+, and Zn^{2+} were strong urease inhibitors; however, they did not inhibit *Arthrobacter* urease. In conclusion, pure urease

can be produced from bacteria and can be used to lower uric acid levels and assay uric acid in blood or urine.

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