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Synthesis and characterization of lactones by *Azotobacter chroococcum*

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Abstract. *The current paper deals with new metabolites of different groups produced by the* Azotobacter chroococcum *XU1 strain. Until now, a wide variety of secondary metabolites were documented for this species, but some compounds are being reported for the first time. These compounds—representatives of lactones. An important finding within this survey was the production of lactones, namely 1,5-D-gluconolactone and D, L-mevalonic acid lactone. It is interesting to note that the strain produced 1,5-D-gluconolactone as a response to the substrate modification (C-source): in the D-glucose supplemented Ashby, the major compound was 1,5-D-gluconolactone instead of EPS (which is produced in the sucrose supplemented Ashby).*

Keywords: *A. chrooccoccum, lactones, 1,5-D-gluconolactone.*

Introduction

Azotobacter chroococcum is a nitrogen-fixing rhizobacterium of the *Azotobacter* genus of the *Pseudomonasaceae* family [1]. As other representatives of the genus, the bacterium also produces physiologically active compounds like indole compounds like indole-3-acetic acid (IAA) and other auxins [2, 3], gibberellins, cytokinins, aminoacids [4], vitamins [5], and a wide variety of other compounds. In recent years, bacteria of this genus have been reported for several EPS and alginate productions [6, 7]. *Azotobacter* ESP has been exploited for different purposes, such as heavy metal biosorption [8] and biofabrication of nanobiomaterials [9, 10]. Besides, it is known that *Azotobacter* EPS is used for salt-stress mitigation [11] and as a biocontrol agent [12, 13]. Apart from these, bacteria of the genus are successfully employed as phosphate mobilizing agents, enhancing phosphorus uptake by plants [14]. Among *Azotobacter* bacteria, basically, two species—*Azotobacter chroococcum* and *Azotobacter vinelandii*—are the most widely employed bacteria in agriculture for crop yield enhancement, biological control, and soil fertility improvement [15]. In this research, we aimed to investigate lactones of a diazotrophic strain *A. chroococcum* XU1.

Materials and methods

Microbial strains, identification and media for cultivation

A. chroococcum XU1 was used throughout the study.

For molecular-genetic characterization, a protocol documented elsewhere [16] was applied in the current research.

For the cultivation of *A. chroococcum* XU1, Ashby broth was used: sucrose, 20 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; KH_2PO_4, 0.2 g/L; NaCl, 0.1 g/L; $CaCO_3$, 10 g/L. The initial pH value of the medium was adjusted to 7.0. Each flask was inoculated with 4% (v/v) of the seed culture and incubated at 30^0C with shaking at 150 rpm for three days [9].

Production, isolation and purification of 1,5-D-Gluconolactone

The biosynthesis of 1,5-D-Gluconolactone by the strain was carried out in a shaker in 2 l flasks containing 1 l of Ashby medium, supplemented with D-Glucose as a single carbon source,

at 150 rpm at 30◦C under intensive aeration. The initial pH value of the medium was adjusted to 7.0. Each flask was inoculated with 4% (v/v) of the seed culture and incubated at 30^0C with shaking at 150 rpm for 3 days. The culture broth was centrifuged at 10,000 rpm for 15 minutes to separate the cells, which were then washed twice with distilled water. The released crude 1,5-D-Gluconolactone was precipitated by the addition of 3 volumes of ice-cold 95% (v/v) ethanol to the supernatants.

Other lactones synthesis by A. chroococcum XU1 *and their extraction*

Evaluation of lactones was carried out as described by Mohamad et al. (2018) with slight modification [17]. Production of lactones by *A. chroococcum* XU1 was carried out in 500 mL^{-1} of broth medium at 28^0C for 12 days with agitation at 120 rpm. The cell biomass was collected by centrifugation at 5000 rpm for 10 min. The cell free supernatant was divided into equal volumes. After that, the supernatant was adjusted to pH 7 and pH 3 with 500 mL^{-1} of 1 N HCl and an equal volume (1:1) of ethyl acetate was added and mixed by vigorous shaking for 30 min and allowed to settle. The organic solvent phase was collected and evaporated at 40^0C under vacuum, using a rotary evaporator model (IKA, HB10 basic). The ethyl acetate extract was dissolved in 5 mL of Tris–Cl buffer (0.02 M, pH 7.0) and used for gas chromatography/mass spectrometry (GC-MS).

NMR of 1,5-D-Gluconolactone

1H, 13C and 2D (HSQC, HMBC, NOESY) NMR spectra were recorded on a Varian-400MR spectrometer (Varian, USA, 400 MHz for 1H and 100 MHz for 13C NMR) in D_2O with DSS as the internal standard.

Results

Identification of bacterial and fungal strains

A partial sequence of 16S RNA for the bacterium and 18S RNA for the fungus identified the strains as *Azotobacter chroococcum* (Fig. 1).

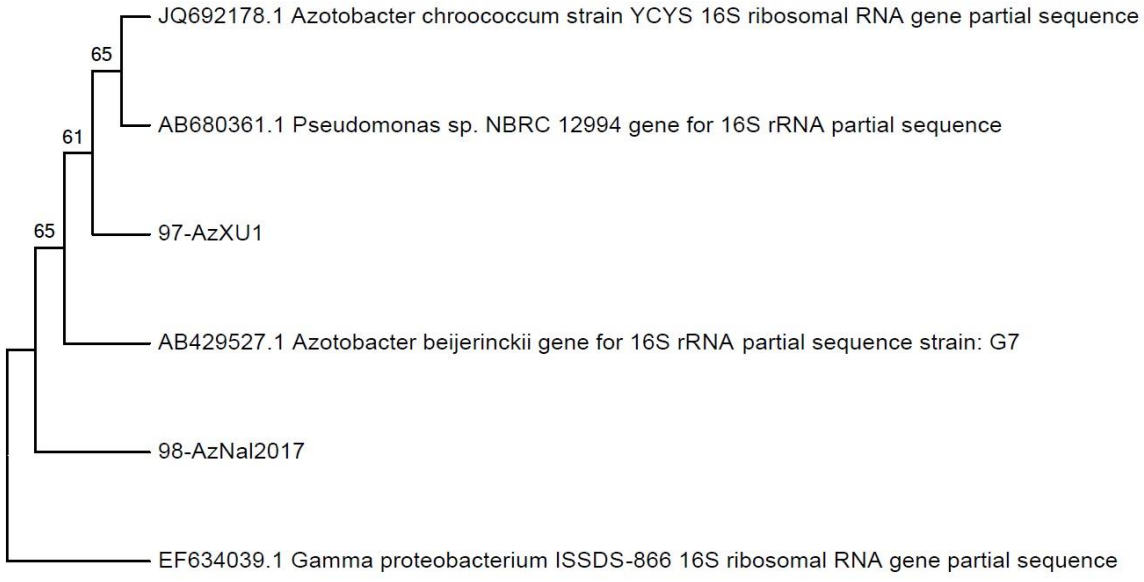

Fig. 1. Phylogenetic placement of *A. chroococcum* XU1 on the basis of partial sequencing of 16S RNA gene

Synthesis and characterization of 1,5-D-gluconolactone

After three days of incubation under intensive aeration and agitation, *A. chroococcum* XU1 in the D-glucose supplemented Ashby broth produced 1,5-D-gluconolactone, and the overall yield of the lactone averaged 6.5–7.0 g/L. It was the only major compound formed during cultivation, and precipitation with ice-cold absolute ethanol resulted in pelleting a highly pure final product: lactone. The lactone was subjected to NMR without any further procedures. Analysis revealed that the lactone was 1,5-D-gluconolactone (Fig. 2, 3; Table 1).

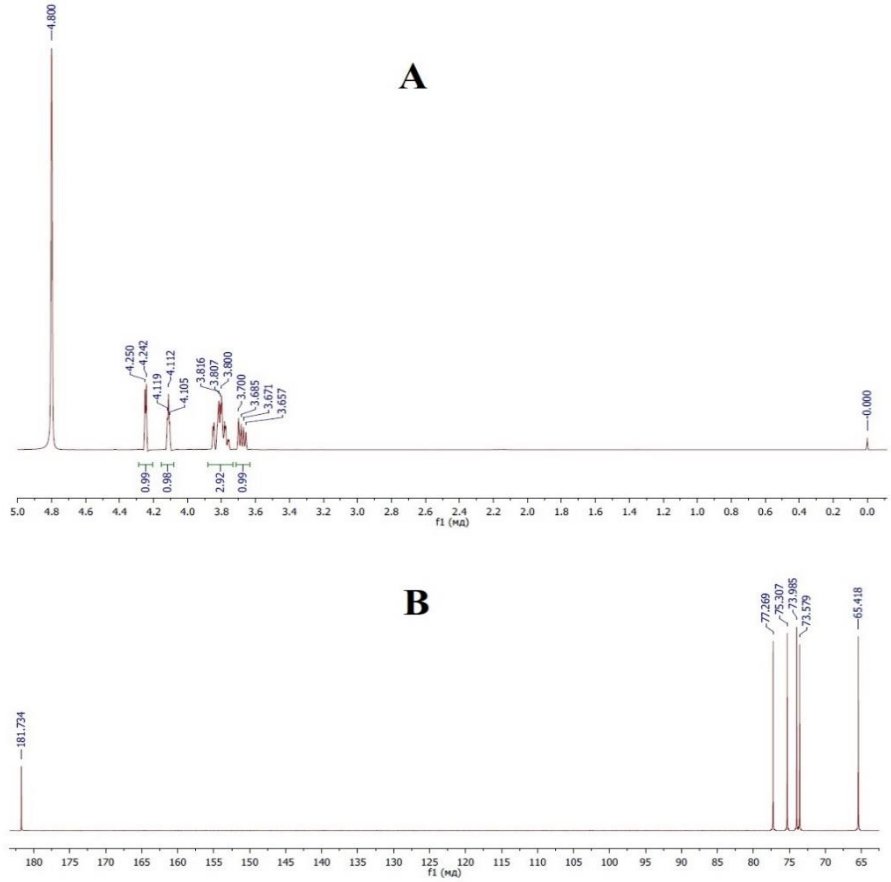

Fig. 2. NMR spectra (A – proton, B – proton) of 1,5-D-Gluconolactone of *A. chroococcum* XU1

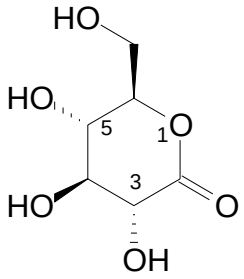

Fig. 3. Chemical structure of 1,5-D-gluconolactone ($C_6H_{10}O_6$) produced by *A. chroococcum* XU1

It is interesting to note that in the sucrose-supplemented Ashby broth, the strain produced alginate-based EPS, but another representative of the lactones, namely, DL-mevalonic acid lactone, was produced in minor quantity among other metabolites of the bacterium. The

replacement of basic C sources in the medium (D-glucose and sucrose) had a significant impact on the final major metabolite (lactone and EPS).

Table 1. ^{1}H and ^{13}C NMR chemical shifts of the compound and data of HMBC, NOESY (D_2O, DSS - 0 ppm), δ, ppm, J/Hz, 400MHz)

C atom	δ_C	δ_H (J/Hz)	HMBC (H)	NOESY
2	181.73		3	
3	77.27	4.25, d, (*J*= 3.2)		
4	73.58	4.11, br.t, (*J*= 3.0)		
5	73.99	3.79, m	6, 7	3
6	75.31	3.80, m	5, 7	4
7	65.42	3.83, dd, (*J*= 11.4; 2.7)	6	
		3.68, dd, (*J*= 11.4; 5.8)		

Bacteria of the *Azotobacter* genus, basically the first two representatives, *A. chroococcum* and *A. vinelandii,* have been widely documented for alginate-based exopolysaccharides as the main major compounds [6, 7]. In our experiments, the C source was a decisive factor for the formation of 1,5-D-gluconolactone (Fig. 2, 3; Table 1). In D-glucose-supplemented Ashby, the strain produced 1,5-D-gluconolactone. We hypothesized that the presence of D-glucose initiates the formation of 1,5-D-gluconolactone by *Azotobacter chroococcum* XU1, replacing that of EPS.

It was hypothesized that these lactones might be auxiliary metabolites of the bacterial cell to respond to either abiotic or biotic stress.

Summarizing the above, *A. chroococcum* XU1 can be effectively exploited as a biofertilizer in salt-affected soils for crop productivity enhancement and phytopathogen biocontrol.

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