Appl Biochem Biotechnol (2011) 164:318–337
DOI 10.1007/s12010-010-9137-3

The Correlations Between TCA-Glyoxalate Metabolite and Antibiotic Production of *Streptomyces* sp. M4018 Grown in Glycerol, Glucose, and Starch Mediums

Leman Tarhan · Hulya Ayar Kayalı · Anıl Sazak · Nevzat Şahin

Received: 20 July 2010 / Accepted: 22 November 2010 /
Published online: 4 December 2010

Abstract The alterations of organic acids citrate, α-ketoglutarate, succinate, fumarate, malate production together with isocitrate lyase activity as a glyoxalate shunt enzyme, and antibiotic production of *Streptomyces* sp M4018 were investigated in relation to changes in the glucose, glycerol and starch concentrations (5–20 g/L) after identification as a strain of *Streptomyces hiroshimensis* based on phenotypic and genotypic characteristics. The highest intracellular citrate and α-ketoglutarate levels in 20 g/l of glucose, glycerol, and starch mediums were 399.47±4.78, 426.93±6.40, 355.84±5.38 ppm and 444.81±5.12, 192.96±2.26, 115.20±2.87 ppm, respectively. The highest succinate, malate, and fumarate levels were also determined in 20 g/l of glucose medium as 548.9±11.21, 596.15±8.26, and 406.42±6.59 ppm and the levels were significantly higher than the levels in glycerol and starch. Extracellular organic acid levels measured also showed significant correlation with carbon source concentrations by showing negative correlation with pH levels of the growth medium. The antibiotic production of *Streptomyces* sp. M4018 was also higher in glucose medium as was the case also for organic acids when compared with glycerol. On the other hand, there is no production in starch.

Keywords *Streptomyces* sp. · TCA cycle · Glyoxalate cycle · Antibiotic production

Introduction

Streptomyces are Gram-positive soil eubacteria with high guanin + cytosin content [1] and are of utmost importance from an environmental, clinical, and industrial standpoint. They produce a diverse range of secondary metabolites that has high impact in production of

L. Tarhan (✉) · H. A. Kayalı
Science Faculty, Department of Chemistry, Dokuz Eylul University, 35150 Buca, Izmir, Turkey
e-mail: leman.tarhan@deu.edu.tr

A. Sazak · N. Şahin
Science and Arts Faculty, Department of Biology, Ondokuz Mayıs University, 55139 Kurupelit, Samsun, Turkey

antibiotics. In the course of screening for new metabolites, several studies were carried out in order to isolate new *Streptomyces* species from different habitats. The metabolite production is regulated by precursors for the synthesis of secondary metabolites which are produced with metabolites of the central metabolism. Metabolic energy is distributed between two kinds of demands: biosynthetic processes that produce a net increase in biomass, and the other processes that regulate the internal pH and osmotic pressure, the macromolecular turnover, the membrane energization, and the motility. A common assumption is that the rate of energy demands of biosynthetic processes changes in a continuous manner with specific growth rate while the energy demand of maintenance processes remains constant [2]. Thus, maintenance energy is an increasingly greater fraction of the total energy flow in the cell at low growth rates and growth efficiency declines. However, the concept of a constant maintenance energy requirement has been repeatedly challenged in recent years [3]. Variations in maintenance requirements have often been difficult to explain but are taken as evidence that the bacteria often consume large amounts of energy in reactions that are not directly related to growth, particularly when growth itself is constrained [4]. The majority of organic acids produced by microorganism can be readily placed into two main groups, depending on their metabolic origin and from the main metabolite sequence of aerobic organism, in the tricarboxylic

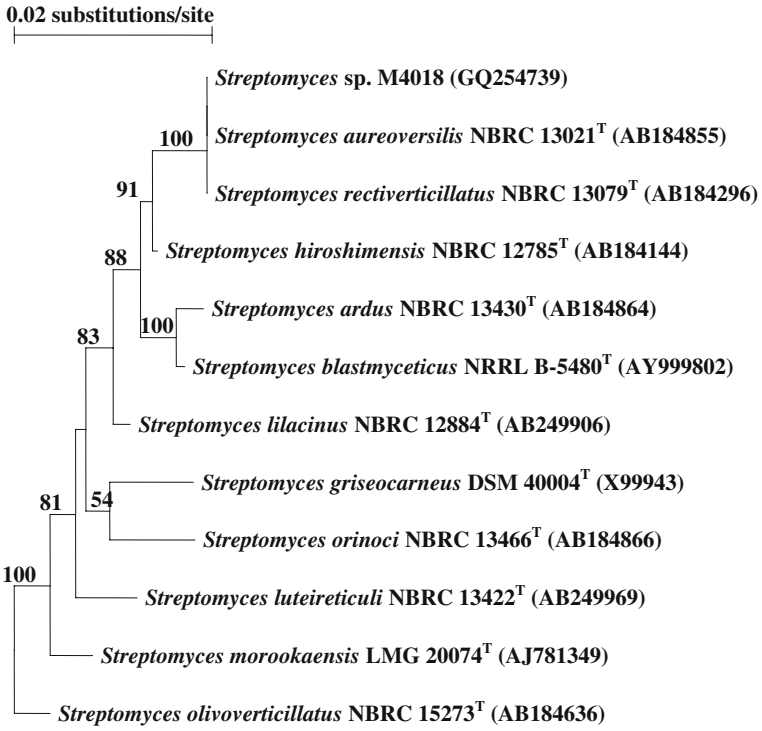

Fig. 1 Neighbor-joining tree [6] showing relationships between *Streptomyces* sp. M4018 and representatives of the *Streptomyces* strain s based on almost complete 16S rRNA gene sequences. *Scale bar* 0.02 substitutions per site. *Numbers at nodes* levels of bootstrap support (%) based on a neighbor-joining analysis of 1,000 resampled datasets; only values >50% are given. NCBI accession numbers are given in *parentheses*

Table 1 Similarities and dissimilarities among 16S rRNA sequences of *Streptomyces* sp. M4018 and *Streptomyces*-type strains

	Streptomyces sp. M4018	*S. rectiverticillatus* NBRC 13079^T	*S. aureoversilis* NBRC 13021^T	*S. olivoverticillatus* NBRC 15273^T	*S. morookaensis* LMG 20074^T	*S. orinoci* NBRC 13466^T	*S. luteireticuli* NBRC 13422^T	*S. blastmyceticus* NRRL B-5480^T	*S. griseocarneus* DSM 40004^T	*S. ardus* NBRC 13430^T	*S. lilacinus* NBRC 12884^T	*S. hiroshimensis* NBRC 12785^T
Streptomycin sp. M4018	–	0	0	31	28	28	30	16	29	18	15	8
S. rectiverticillatus NBRC 13079^T	100.00	–	0	31	28	28	30	16	29	18	15	8
S. *aureoversilis* NBRC 13021^T	100.00	100.00	–	31	28	28	30	16	29	18	15	8
S. *olivoverticillatus* NBRC 15273^T	97.87	97.87	97.87	–	16	32	22	23	31	26	21	22
S. *morookaensis* LMG 20074^T	98.08	98.08	98.08	98.90	–	22	18	23	29	25	19	19
S. *orinoci* NBRC 13466^T	98.08	98.08	98.08	97.81	98.49	–	27	26	23	30	20	25
S. *luteireticuli* NBRC 13422^T	97.94	97.94	97.94	98.49	98.76	98.15	–	25	21	26	16	20
S. *blastmyceticus* NRRL B-5480^T	98.90	98.90	98.90	98.42	98.42	98.22	98.28	–	32	5	14	8
S. *griseocarneus* DSM 40004^T	98.01	98.01	98.01	97.87	98.01	98.42	98.56	97.80	–	35	21	27
S. *ardus* NBRC 13430^T	98.76	98.76	98.76	98.21	98.28	97.94	98.21	99.66	97.59	–	16	10
S. *lilacinus* NBRC 12884^T	98.97	98.97	98.97	98.56	98.69	98.62	98.90	99.04	98.55	98.90	–	6
S. *hiroshimensis* NBRC 12785^T	99.44	99.44	99.44	98.46	98.67	98.25	98.60	99.44	98.11	99.30	99.58	–

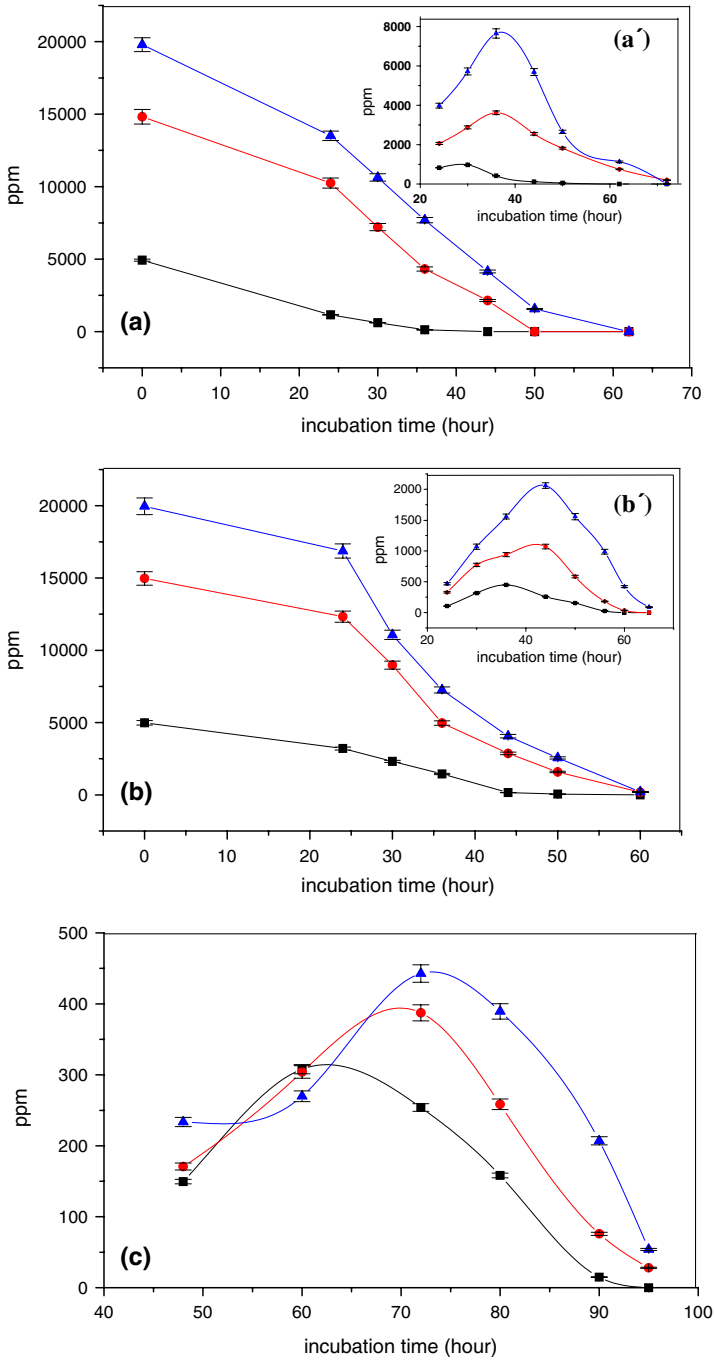

Fig. 2 Variation in the extra- and intra-cellular glycerol and glucose levels of *Streptomyces* sp. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

acid cycle (TCA) cycle and glycolysis and acid arising from the oxidation of glucose [5]. The second group produced by only one or two enzymatic steps using glucose is in this respect important in biotransformation to produce organic acids. Another carbon source, glycerol is metabolized by the glycerol dehydrogenase which catalyzes the conversion of glycerol to dihydroxyacetone and the glycolytic enzyme dihydroxyacetone kinase phosphorylates the latter product and then funneled to glycolysis [7]. In addition, numerous bacteria including genus *Streptomyces* are able to secrete starch-degrading enzymes such as amylases and thus, to use starch as sole carbon and energy source [8].

Organic acids and metabolites play an important role in food, chemical, and pharmaceutical industries [9]. Consequently, the study of microbial organic acid production is gaining increased interest [10]. The majority of organic acids are produced by microorganism depending on their metabolic sequence of aerobic organism. The efficiency of organic acid accumulation by microorganism depends on various parameters. Among these, the type and the concentration of the sugar consumed have high impact on acid production, while the optimal concentration of the trace metals, phosphate, and nitrogen are interrelated.

The aim of the present study is to examine the relationship between the production of citrate, α-ketoglutarate, succinate, fumarate, malate as TCA cycle organic acids and isocitrate lyase as a glyoxalate shunt enzyme, and antibiotic of *Streptomyces* sp. M4018, which was firstly isolated and identified, grown in various concentrations of glucose, glycerol, and starch as carbon sources during the incubation period.

Materials and Methods

Isolation and Phenotypic Characterization

Strain M4018 was isolated from the rhizosphere samples of the *Colutea arborescens* on starch casein agar [11] supplemented with filter sterilized cyclohexemide (50 μg/ml), nystatin (50 μg/ml), and rifampicin (0.5 μg/ml) incubated at 28 °C for 14 days. The isolate was grown on oatmeal and peptone–yeast extract–iron agar [12] plates at 28 °C for 14 and 4 days, respectively. Aerial spore mass color, substrate mycelium pigmentation, and the color of any diffusible pigment were recorded on oatmeal agar. The peptone yeast extract–iron agar plates were examined to see whether the strain produced melanin pigments.

Spore chain morphology and spore surface ornamentation were examined by scanning electron microscopy of preperations from culture grown on oatmeal agar for 2 weeks at 28 °C, following the procedure described by O'Donnel et al. [13].

The isomers of LL-diaminopimelic acid (A_{2pm}) was determined by thin-layer chromatography of hole-organisms hydrolysates on cellulose acetate sheats following the procedure described by Staneck and Roberts [14], but using modified solvent system methanol/H_2O/HCl/10N pyridine (85:15:5:10, *v*/*v*).

Menaquinones were extracted and purified following the method of Collins [15] and then analyzed by high-performance liquid chromatography (HPLC) [16]. Fatty acids were extracted, methylated and analyzed by GC using the standart MIDI system (Microbial Identification; Microbial ID, Inc. Newark, Del.) [17, 18].

DNA G + C content of the strain was obtained using the thermal denaturation method of Gonzalez and Saiz-Jimenez [19] with *Escherichia coli* 1.365.

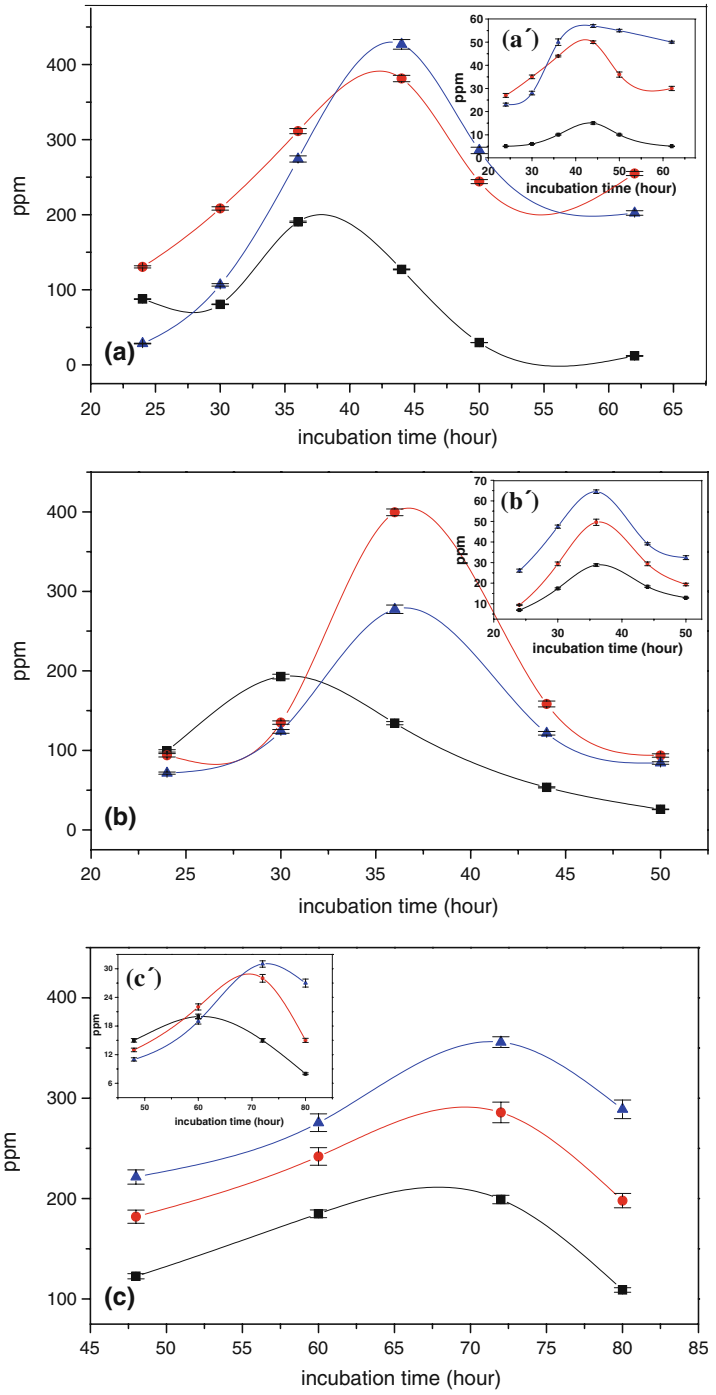

Fig. 3 Variation in the intra- and extra-cellular citrate levels of *Streptomyces sp*. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), and 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

Chromosomal DNA Isolation and PCR Amplification

The guanidine thiocyanate DNA extraction procedure of Pitcher was used to isolate DNA from test strains [20]. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using a prokaryotic universal primers: p27f (5′-AGA GTT TGATCC TGG CTC AG 3′) and pl525r (5′-AAG GAG GTG WTC CAR CC 3′) [21, 22] and HotStar *Taq*® DNA polymerase (Qiagen). The amplified fragments were purified with QIAquick purification kits (Qiagen, Valencia, USA).

Sequencing of 16S rDNA and Phylogenetic Analysis

Purified PCR products sequenced directly using ABI PRISM^{R} BigDye™ Terminator Cycle Sequencing kits (Applied Biosystems). The sequence of the complete lengths of the 16S rRNA gene of isolate was determined in separate cycles using two internal forward primers (27f and MG3f) [21, 22] and one reverse oligonucleotide primer (1525r) [23]. Sequencing gel electrophesis was carried out and the nucleotid sequences were automatically obtained by using an Applied Biosystem DNA sequencer system (model 310) and software provided by the manufacturer. The two forward and the single reverse complementary 16S rRNA gene sequences were manually assembled using the PHYDIT program, version 1 [22], and aligned against corresponding sequences of representatives of *Streptomyces* retrived from Genbank (NCBI, USA).

Phylogenetic trees and similarity matrices were constructed using the PHYLIP suite of programs [24]. The evolutionary distance model of Jukes and Cantor [25] was used to generate evolutionary distance matrices for the neighbor-joining algorithm. The topologies of the resultant trees were evaluated in a bootstrap analysis [26] based on 1,000 resampling of the neigbor-joining dataset using the CONSENSE and SEQBOOT options from the PHYLIP package. The 16S rRNA gene sequences determined for the *Streptomyces* sp. M4018 deposited in GenBank database, under the accession number GQ254739.

Media and Growth Conditions

Spore cultures of *Streptomyces* sp. M4018 were prepared by inoculating solid medium M65 which contained 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$, 12 g Agar, and 20 g starch in 1 l of ultra-pure water [27]. The basal chemically defined fermentation medium was consist of 0.6 g $MgSO_4.7H_2O$, 3.5 g KH_2PO_4, 2.0 g asparagine, 10 g glycerol, 21.0 g 3-(N-morpholino) propanesulfonic acid buffer, and 1 ml trace salts solution (1.0g $FeSO_4.7H_2O$, 1.0g $MnCl_2.4H_2O$, 1.0g $ZnSO_4.H_2O$, 1.0g $CaCl_2$) in 1 l of ultra-pure water. The pH level was set to 7.0 before autoclaving. The cultures were inoculated with 2 ml spore suspensions and incubated with agitation at 150 rpm at 28 °C in 500-ml shaking flasks containing 50 ml of culture for 96 h. After the cultivation process, the cells were harvested by centrifugation followed by washing twice with distilled water and kept at −20 °C.

Preparation of Cell-Free Extracts

The *Streptomyces* sp. M4018 cells were harvested by centrifugation, washed twice with 10 mM potassium-phosphate buffer pH 7.5, containing 2 mM EDTA, and stored at −20 °C. Before assaying, the samples were thawed, washed, and resuspended in 100 mM potassium-phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol in a volume equal to 1.5 times

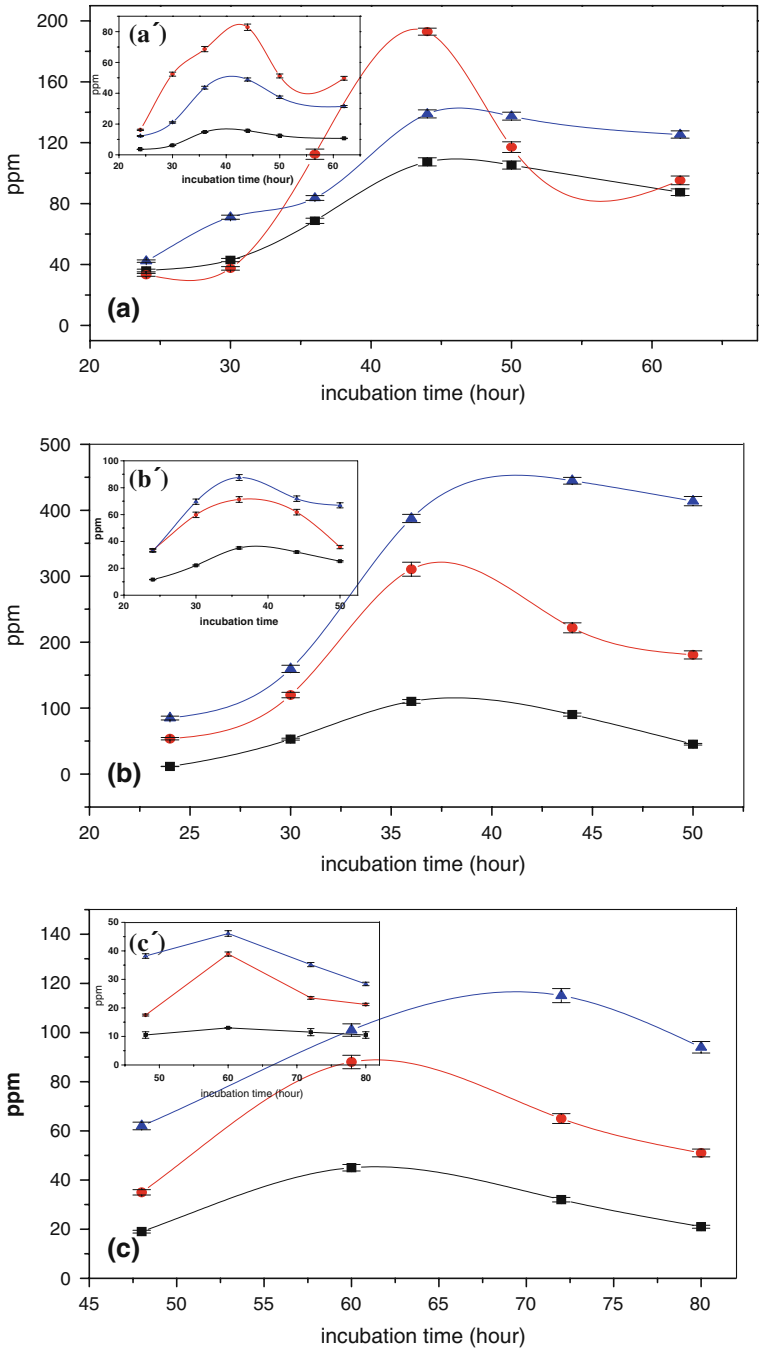

Fig. 4 Variation in the intra- and extra-cellular α-ketoglutarate levels of *Streptomyces* sp. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

its weight. A 600-μl cell suspension was ground in 1.5-ml plastic vials with 0.6 g of glass beads (0.25 mmφ) for 10 min. Cell debris was removed by centrifugation at 15,000 rpm for 15 min. The cell free extracts were kept at −20 °C until they were used for HPLC analysis and isocitrate lyase activity measurements. One milliliter of culture was centrifuged and the supernatant was then filtered through a 0.45-μm syringe filter for HPLC analysis.

Organic Acid Analysis

The used HPLC system was equipped with Alltech IOA-1,000 column, a UV detector, and a differential refractive index detector (RI). Applied to the column operated at 42 °C was 0.4 ml/min mobile phase using 9.0 mM H_2SO_4 solution. Standards were prepared for glucose, α-ketoglutarate, citrate, malate, succinate, fumarate for both the RI detector and UV detector (210 nm), and calibration curves were created.

Assay of Isocitrate Lyase Activity

Isocitrate lyase was assayed mixture for isocitrate lyase contained, in 2 ml, 15 μmol of $MgCl_2.6H_2O$, 10 μmol of phenylhydrazine-HCl, and 6 μmol cysteine-HCl. The reaction for the assay was initiated by adding crude cell-free extract to be assayed and 0.2 ml or 5 μmol of trisodium dl-isocitrate. The formation of glyoxylic acid phenylhydrazone was followed by monitoring absorbance at 324 nm. The enzyme activity was expressed as U/mg protein, with 1 unit enzyme activity equal to 1 nmol glyoxalate formation [28].

Statistical Analysis

Tukey's test, one of the multiple comparisons, was used for statistical significance analyses. The values are the mean of three separate experiments. Also, comparison between enzyme activities, organic acid levels of the culture medium were carried out using *Pearson correlation* for each incubation time.

Results

The variations of tricarboxylic acid and glyoxalate cycle metabolite levels and enzyme activities such as citrate, α-ketoglutarate, succinate, fumarate, malate, and isocitrate lyase as well as antibiotic production of newly isolated and identified *Streptomyces* sp M4018 by using poliphasic taxonomic approach were also investigated depending on the concentrations of glycerol, glucose, and starch in the growth medium. These three different carbon sources were selected to represent the most abundant and available carbohydrates for the production of chemicals via microbial fermentations.

Isolation, Characterization, and Identification of *Streptomyces* sp M4018

The organism exhibited a range of chemotaxonomic and phenotypic properties typical of members of the genus *Streptomyces*. It formed an extensively branched substrate mycelium, aerial hyphae which carried smooth-surfaced spores in short verticillus spore chains and pink aerial spore mass on several standard media. It contained LL-diaminopimelic acid in whole-organism hydrolysates. Fatty acid analysis showed that strain M4018 consisted of a

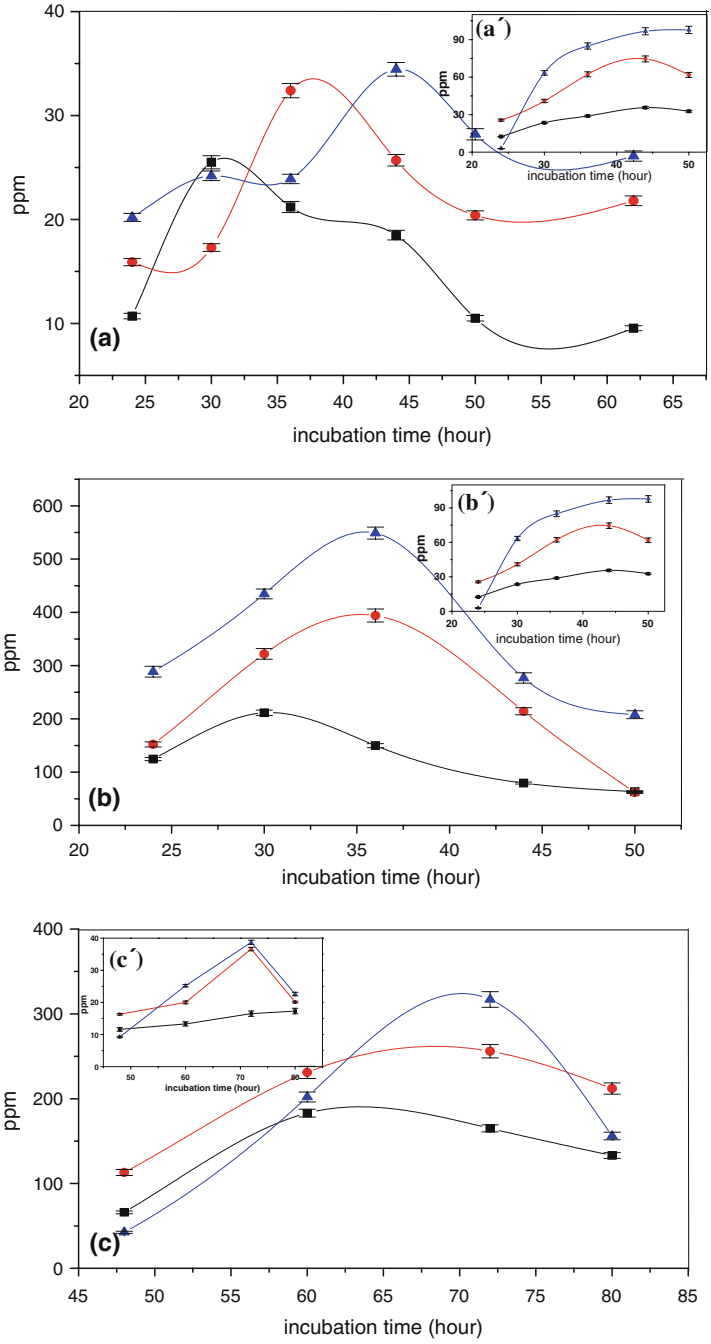

Fig. 5 Variation in the intra- and extra-cellular succinate levels of *Streptomyces sp*. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

high proportion of saturated straight chain, iso- and anteiso-branched fatty acids. The predominant cellular fatty acids were 12-methyltetradecanoic acid (anteiso-$C_{15:0}$), 14-methylpentadecanoic acid (iso-$C_{16:\ 0}$), and hexadecanoic acid ($C_{16:\ 0}$). The organism also consisted of hexahydrogenated menaquinone with nine isoprene units as the predominant isoprenologues. The G + C content of the genomic DNA was 71.2 mol%. An almost complete 16S rRNA gene sequence (1,524 nt) was determined for the organism. Primary sequence analysis with the sequences of representatives of the family *Streptomycetaceae* confirmed that the unknown isolate was closely related to the species of the genus *Streptomyces* (Fig. 1). The highest 16S rRNA gene sequence similarity value was determined as 99.44% (with 8 nt differences) for *Streptomyces hiroshimensis* NBRC 12785^T (Table 1). However, strain M4018 showed 100% 16S rRNA gene sequence similarity value with *Streptomyces rectiverticillatus* NBRC 13079^T (AB184296) and *Streptomyces aureoversillis* NBRC 13021^T (AB184855) which were described as the synonym of the type strain of *S. hiroshimensis* by Hatano [29]. Based on phenotypic, chemotaxonomic, and phylogenetic results, it was possible to identify *Streptomyces* sp. M4018 as a strain of *S. hiroshimensis*.

The Variations of Extra- and Intracellular Carbon Sources of *Streptomyces* sp M4018

As presented in Fig. 2a, a complete consumption of extracellular glycerol in *Streptomyces* sp. M4018 growth medium occurred at 30th hour for 5 g/l and at 50th hour for 15 and 20 g/l of glycerol ($p<0.01$). The consumption time was 44 h for 5 g/l and 60 h for 15 and 20 g/l by delaying for glucose. Nonetheless, it was observed that decreasing rates of extracellular glycerol and glucose were higher levels after 24th hour of incubation except for 5 g/l of glycerol ($p<0.01$).

The intracellular glycerol and glucose levels increased with the increasing carbon source concentrations in the growth medium by showing positive correlation with the biomass levels. The maxima were determined at 30th for 5 g/l; 36th hours for 15 and 20 g/l of glycerol and at 36th and 44th hours for the same concentration of glucose ($p<0.01$; Fig. 2a′, b′). The intracellular glycerol and glucose levels showed significant negative correlation with extracellular levels, respectively ($r=-0.738$ and -0.768 $p<0.01$).

The intracellular glucose level of *Streptomyces* sp. M4018 grown in starch medium also increased with increases in starch concentrations (Fig. 2c). The maximum intracellular glucose level was determined at 60th hour for 5 g/l while it shifted to 72nd hour for 15 and 20 g/l of starch. The increases in intracellular glucose level were lower than glycerol and glucose supplemented mediums by showing positive correlation with the cell density variations ($p<0.01$).

The Variations of TCA Metabolites and Isositrate Lyase Activity of Glyoxalate Cycle Enzyme of *Streptomyces* sp M4018

As an important metabolite in TCA, the intracellular citrate level of *Streptomyces* sp. M4018 increased with increase in concentrations of glycerol and starch while it rose up to 15 g/L for glucose (Fig. 3a–c,) ($p<0.01$). The maximum intracellular citrate level in glycerol, glucose, and starch mediums were determined at 36th, 30th, and 60th hours for 5 g/l while they were shifted to 36th, 44th, and 72th hours for 5 and 15 g/L for these carbon sources, respectively. The highest levels in these mediums were 426.93±6.40, 399.47± 4.78, and 355.84±5.38 ppm, respectively.

The extracellular citrate level showed positive correlation with the concentration of glycerol, glucose, and starch ($r=0.779$, $p<0.01$; $r=637$ and 0.554; $p<0.05$; Fig. 3a′, b′, c′).

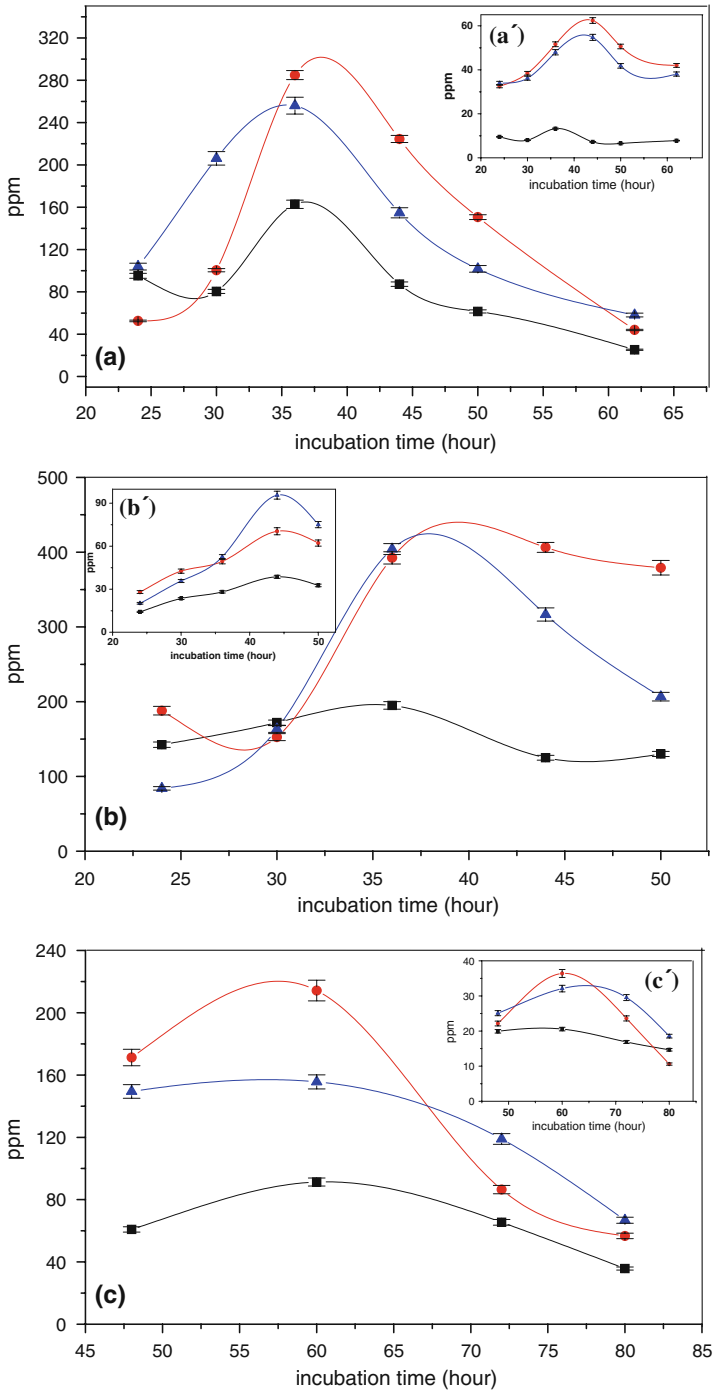

Fig. 6 Variation in the intra- and extra-cellular fumarate levels of *Streptomyces sp*. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

The extracellular citrate level reached its maximum at 60th hour for 5 g/l and 72nd hour for 15 and 20 g/l of starch while the maximum was occurred at the 44th hour and the 36th hour for all studied concentrations of glycerol and glucose, respectively.

As seen in Fig. 4a, the intracellular α-ketoglutarate level of *Streptomyces* sp. M4018 increased with increasing glycerol concentration up to 15 g/l of the culture medium. This show significant positive correlation with the concentration of both glucose and starch (r= 0.819 and 710; $p<0.01$; Fig. 4b, c). The maximum intracellular α-ketoglutarate level of *Streptomyces* sp. M4018 was observed at 44th hour for all studied concentration of glycerol; and at 36th hour for 5,15, and at 44th hour for 20 g/l of glucose. The level was shifted to 60th hour for 5, 15 g/l and 72nd hour for 20 g/l of starch as was the case in citrate levels. The highest intracellular α-ketoglutarate level was 444.81±5.12 ppm for glucose, 192.96±2.26 ppm for glycerol, and 115.20±2.87 ppm for starch-supplemented medium.

The extracellular α-ketoglutarate level showed a positive correlation with intracellular level depending on the concentrations of the glycerol, glucose and starch (r=0.745, 0.867, 0.866; $p<0.01$; Fig. 4a′, b′, c′). The extracellular α-ketoglutarate level reached its maximum at 44th, 36th, and 60th hours in glycerol, glucose, and starch mediums ($p<0.01$) and they were 82.86±2.07, 87.59±2.19, and 46.1±0.96 ppm, respectively.

Succinic acid is produced via the action of anaplerotic enzymes and the TCA-reductive arm by microorganism. As can be seen from Fig. 5a–c; the intracellular succinate level correlated positively with concentrations of glycerol, glucose, and starch (r=0.804, 0.647, $p<0.01$; 0.598, $p<0.05$). This level at *Streptomyces* sp. M4018 grown in glycerol, glucose, and starch mediums reached its maximum at the 36th, 30th, and 60th hours at 5 g/l. However, it was shifted to 44th, 36th, and 72nd hours for 15 and 20 g/l, respectively ($p<0.01$). The highest level were 394±7.88, 548±11.21, and 317±9.19 ppm for glycerol, glucose, and starch mediums, respectively.

Extracellular succinate levels of *Streptomyces* sp. M4018 increased with the increase in the carbon concentration, showing a parallelism with the intracellular levels ($p<0.01$; Fig. 5a′, b′, c′). The incubation time of the measured maximum level were 44th and 72nd hours for all used glucose and starch concentrations and shifted to right in the range of 30th to 44th hours by increase in glycerol concentration. The highest level was determined at 20 g/l of glucose as 96.70 ppm which is 2.8- and 2.5-fold higher than the levels of glycerol and starch.

Fumaric acid, widely used in industrial applications, was produced from *Streptomyces* sp. M4018 for glycerol. The intracellular fumarate level increased with increasing concentration of all used carbon sources up to 15 g/l (Fig. 6a–c). The intracellular fumarate level in glycerol and glucose mediums reached its maximum at 36th hour ($p<0.01$) while it was at 60th hour in starch medium. The highest fumarate level was determined in glucose medium as 406.42±6.59 ppm and the level was significantly higher than the levels in glycerol and starch, as was the case also for other citrate cycle metabolites ($p<0.01$).

Extracellular fumarate level in glycerol and glucose mediums reached its maximum at 44th hour except for 5 g/l of glycerol (Fig. 6a′, b′). It was at 60th hour for all used starch concentrations (Fig. 6c′). The highest extracellular level in glucose medium was 95.60±2.77 ppm.

The intracellular malate level of *Streptomyces* sp. M4018 increased with the increase in the concentration of glycerol, glucose, and starch (r=0.630, 0.689, 0.852; $p<0.01$; Fig. 7a, b, c). The maximum intracellular malate level in glucose medium was at 30th hour for 5 g/l and 36th hour for 15 and 20 g/l while it was more dispersed with maxima shifting to the right to longer periods for glycerol and starch at 44th hour and 72nd hour, respectively ($p<0.01$). The highest malate level in glucose, glycerol and starch was 596.15±8.26, 479.99±6.41, and 395.37± 7.25 ppm, respectively.

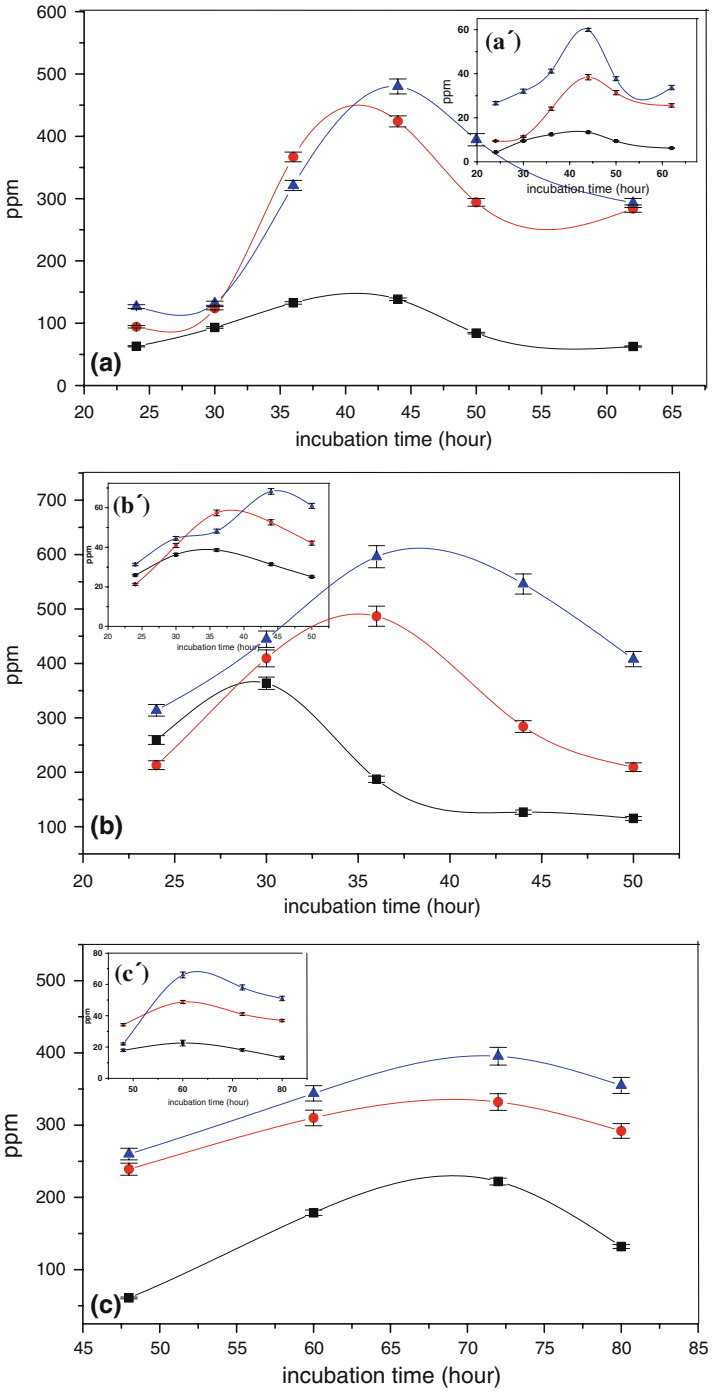

Fig. 7 Variation in the intra- and extra-cellular malate levels of *Streptomyces sp*. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

There was a positive correlation between the extracellular malate level and the glucose, glycerol and starch concentrations showing similar trend to that of intracellular levels (r=0.791, p<0.01; 0.587, p<0.05; 0.791, p<0.01). In addition, these levels of *Streptomyces* sp. M4018 growth in glycerol and starch mediums reached the maximum values on 44th and 60th hours while they were at 36th hour for 5 g/l and 44th hour for 15 and 20 g/l of glucose (Fig. 7a′, b′, c′). pH level variations of *Streptomyces* sp. M4018 growth medium were also showed negative correlation with respect to extracellular organic acid production (p<0.05). The highest decrease was 0.3 pH unit for 20 g/L of both glucose and glycerol while it was only 0.15 pH unit for starch.

Isocitrate lyase activities of *Streptomyces* sp. M4018 showed negative correlation with the increase in the concentration of all studied carbon sources. The activity increased continuously at 5 g/l of glycerol while they decreased up to 36 h for 15 and 20 g/L. Isocitrate lyase activities of *Streptomyces* sp. M4018 grown in five glucose concentration decreased up to 36th while they shifted to 44th hours for and 15, 20 g/L. On the other hand, the activity decreased continuously for all used concentrations of starch during the incubation period. The highest isocitrate lyase activities for the investigated incubation period were 107.0±1.44, 88.6±0.89, and 119.2±1.21 IU/mg for glycerol, glucose, and starch, respectively (Fig. 8).

The Variations of Antimicrobial Activities of *Streptomyces* sp M4018

Peak antimicrobial activities of *Streptomyces* sp. M4018 were investigated for all used carbon sources concentrations depending on the incubation periods. The results showed that the antimicrobial activities of *Streptomyces* sp. M4018 were higher in glucose medium compared with glycerol, but they were undetectable values in starch medium. As can be seen from Table 2, the activities in glucose and glycerol mediums reached their maximum levels at 30th and 44th hours, respectively. Nonetheless, the antimicrobial activities were more effective on the *Pseudomonas aeriginosa*, *Bacillus licheniformis*, *Micrococcus luteus*, and *Saccharomyces cereviciae*, and these efficiencies decreased generally with increases in concentration of both glucose and glycerol.

Discussion

Streptomyces genus is widely recognized as industrially important microorganisms since they exhibit remarkable capacity for the synthesis of secondary metabolites. In the course of screening for new secondary metabolites such as antibiotic, several studies were carried out in order to isolate new *Streptomyces* species from different habitats. The level of antibiotic production depends on the availability of the primer metabolites such as citric acid cycle which also have a wide range of application [30]. Nevertheless, cultural conditions play a major role in enhancing these product formations; one of which is concentration of carbon source [31].

In this study, the taxonomic position of the legume rhizosphere soil isolate, strain M4018, was established using numerical data and an almost complete 16S rRNA gene sequence analysis. Primary sequence analysis with the sequences of representatives of the family *Streptomycetaceae* confirmed that the unknown isolate was closely related to the species of the genus *Streptomyces* and the highest 16S rRNA gene sequence similarity value was obtained for *S. hiroshimensis* NBRC 12785^{T}.

The results of the research demonstrates that the intracellular glycerol and glucose levels of *Streptomyces sp. M4018* are increased with the increases in the carbon source

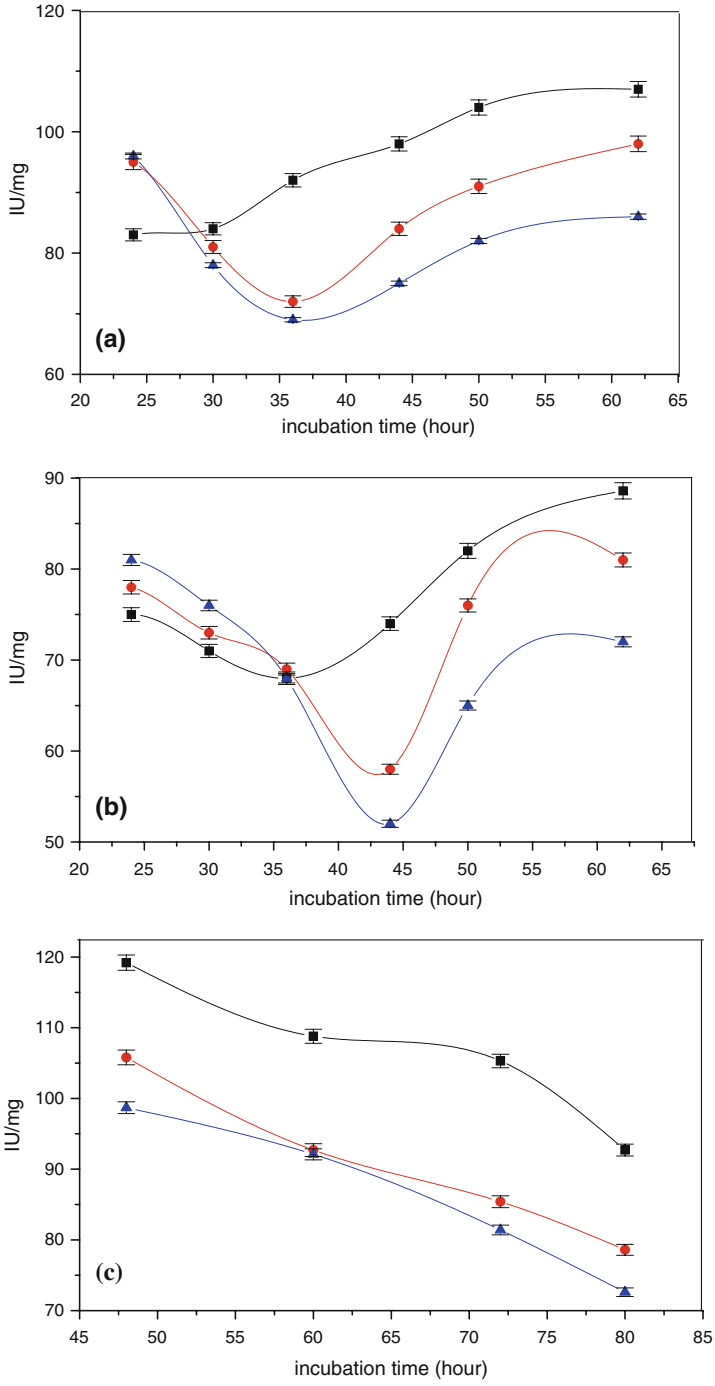

Fig. 8 Variation in the isocitrate lyase activity of *Streptomyces sp*. M4018 depending on incubation periods in medium containing: 5 g/l (*filled square*), 15.0 g/l (*filled circle*), 20 g/l (*filled triangle*) glycerol (**a**), glucose (**b**), and starch (**c**)

concentration in the growth medium. In addition, 2.0-fold higher intracellular glycerol and glucose levels at 20 g/l compared with 15 g/l of carbon sources suggest that consumption rate is significantly higher at 15 g/l in order to maintain energy metabolism [32]. The gradual decrease of the difference between the levels of both intracellular glycerol concentrations indicates that glycerol is consumed in varied metabolic pathways after log-phase of incubation. The maximum consumption of glycerol and glucose in *Streptomyces* sp. M4018 grown in 15 g/l at the time, are 66% and 91%, respectively. The intracellular glucose level in starch supplemented medium is significantly lower than the level in the glucose medium. This may be due to insufficient α-amylase production of *Streptomyces M4018* grown in high starch medium.

As an important TCA cycle metabolite, intracellular citrate level showed positive correlation with the investigated concentration of glycerol and starch in *Streptomyces* sp. M4018 growth medium. This increase shows agreement with some articles that report increase in concentration of carbon sources support higher production of citrate [33]. The intracellular citrate level in *Streptomyces* sp. M4018 growth in glucose medium may increase up to 15 g/l. Further increase in glucose concentration does not enhance citrate production. This may be due to the anaplerosis reaction where pyruvate is transformed into oxaloacetate by a constitutive cytosolic pyruvate carboxylase or repression of citric acid production by citrate synthase which is key enzyme in TCA cycle [34]. When the intracellular citrate level in the studied carbon sources are compared, the maximum citrate level observed in glycerol and glucose are significantly higher than the one determined in starch. This suggests that in high rates of citrate accumulation, easily metabolizable carbon sources such as glycerol or glucose trigger a rise of the intracellular concentration of citrate [35].

In the study, intracellular α-ketoglutarate levels also shows positive correlation with the concentration of glucose and starch in medium while they increased up to 15 g/l of glycerol. This indicates that glycerol is used as a precursor of fatty acid metabolism at high initial glycerol concentration, where glycerol-dehydrating reaction is the major rate-limiting reaction in the reductive pathway [36]. Intracellular succinate and malate levels of *Streptomyces* sp. M4018 increase significantly with the increase in concentration of the carbon sources. Many researchers have also shown that the concentration of the carbon source in growth medium effects the production of primary and secondary metabolites of microorganism [37].

As shown in the results that the intracellular succinate and malate levels for all carbon sources studied are significantly higher than the level of α-ketoglutarate. The increase can be explained by the production of these organic acids in glyoxalate shunt besides citric acid cycle and activated carbon flux into this shunt in the medium containing glycerol and starch which are not sole carbon and energy source [38]. The high intracellular fumarate level in glucose medium indicates that glyoxalate shunt is highly active in glycerol and starch, and a citrate cycle is in glucose medium [39]. This shows coherence with the higher isocitrate lyase activities in starch and glycerol than glucose medium. Nevertheless, decreased activity levels with increase in concentration of all used carbon sources may indicate that glyoxalate shunt is active during starvation of the carbon sources.

The extracellular organic acid level has positive correlation with the concentration of carbon sources while it shows negative correlation with the pH level of the medium. The decrease in pH level is due to increase in the permeability of the cell membrane for the organic acids besides the hydrolyzing products of some nutrients in

Table 2 Variation in the antimicrobial activities of *Streptomyces sp*. M4018 depending on concentrations of glucose and glycerol at 30th and 44th hours

Carbon sources	g/l	Antibiotic activity (mm)									
		Gr (−) bacteria			Gr (+) bacteria				Fungi		Yeast
		1	2	3	4	5	6	7	8	9	10
Glucose	5	14.5±0.5	9.5±0.2	8.5±0.4	16.5±0.3	16.0±0.4	11.0±0.4	10.0±0.3	15.0±0.4	11.0±0.2	11.5 ±0.2
	15	13.0±0.4	10.5±0.4	6.8±0.2	16.8±0.4	13.0±0.5	10.5±0.3	10.0±0.2	14.6±0.5	11.0±0.2	11.0±0.3
	20	13.0±0.5	10.0±0.3	6.1±0.4	17.5±0.2	13.0±0.8	9.6±0.4	9.0±0.2	13.2±0.3	9.5±0.3	11.0±0.4
Glycerol	5	11.5±0.3	10.0±0.6	9.5±0.3	10.0±0.5	9.0±0.2	11.5±0.5	8.0±0.3	12.5±0.3	12.3±0.5	13.0±0.4
	15	9.0±0.6	10.0±0.5	8.0±0.2	10.0±0.2	7.6±0.3	9.5±0.2	9.4±0.4	10.5±0.4	9.5±0.6	10.0±0.5
	20	8.8±0.2	8.5±0.3	8.0±0.5	10.0±0.3	7.5±0.3	9.0±0.2	11.5±0.3	9.5±0.3	7.5±0.5	9.0±0.2

Used strain *1*, *P. aeruginosa* NRRL B-2679; *2*, *P. vulgaris* NRRL B-123; *3*, *E. coli* ATCC 25922; *4*, *B. licheniformis* NRRL B-10015; *5*, *M. luteus* NRRL B-287 *M. luteus*; *6*, *B. subtilis NRRL B-209*; *7*, *S. aureus* ATCC 25923; *8*, *S. cerevicia* ATCC 9763; *9*, *C. albicans* ATCC 10231; *10*, *A. parasiticus* NRRL B-465

growth medium [40]. However, extracellular organic acid level is decreased after reaching its maximum. Further increase in incubation period does not enhance production of these organic acids due to the age of the *Streptomyces* sp. M4018 and depletion of the carbon sources contents in the culture broth [41]. The result supported that after depletion of the favored carbon sources, organic acids are used for metabolite biosynthesis.

According to the results, as a secondary metabolite, the antimicrobial activity of *Streptomyces* sp. M4018 grown in glucose medium is significantly higher than the glycerol as in the case of metabolites of citric acid cycle. In addition, antibiotic productions show parallelism with the log phase periods of *Streptomyces* sp. M4018 grown in glycerol and glucose mediums. Nevertheless, antimicrobial activity is not observed in starch medium probably because of the extended log phase period. All of the results show that the feeding of *Streptomyces* sp. M4018 with glucose maintains the cell metabolism, and thereby enhances the production of the precursors of the antimicrobial metabolites effectively. The result also showed coherence with many published reports that simple sugar such as glucose, fructose, sucrose glycerol enhance growth, the production of intermediates leading to primary as well as secondary metabolite production by microorganisms rather than complex carbon sources like starch, galactase, and xylose. The lowest organic acid level of *Streptomyces* sp. M4018 grown in starch medium suggests that the strain can provide the energy production only to maintain its life and therefore, it cannot produce precursors of antimicrobial metabolites.

References

1. Chater, K. F. (1993). *Annual Review of Microbiology, 47*, 685–713.
2. Gerstmeir, R., Wendisch, V. F., Schnicke, S., Ruan, H., Farwick, M., & Reinscheid, D. (2003). *Journal of Biotechnology, 104*, 99–122.
3. Naeimpoor, F., & Mavituna, F. (2000). *Metabolic Engineering, 2*, 140–148.
4. Theobald, U., Mailinger, W., Baltes, M., Reuss, M., & Rizzi, M. (1997). *Biotechnology and Bioengineering, 55*, 305–316.
5. Ferea, T. L., Botstein, D., Brown, P. O., & Rosenzweig, R. F. (1999). *Proceedings of the National Academy of Science, 96*, 9721–9726.
6. Saito, N., & Nei, M. (1987). *Molecular Biology and Evolution, 4*, 406–425.
7. Zhu, M. M., Lawman, P. D., & Cameron, D. C. (2002). *Biotechnology Progress, 18*, 694–699.
8. Virolle, M. J., & Bibb, M. J. (1988). *Molecular Microbiology, 2*, 197–208.
9. Aristidou, A., & Penttial, M. (2000). *Current Opinion in Biotechnology, 11*, 187–198.
10. Zeikus, J. G., Jain, M. K., & Elankovan, P. (1999). *Applied Microbiology and Biotechnology, 51*, 545–552.
11. Küster, E. (1959). *Int Bull Bacteriol Nomen Taxon, 9*, 97–104.
12. Shirling, E. B., & Gottlieb, D. (1966). *International Journal of Systematic Bacteriology, 16*, 313–340.
13. O'Donnell, A. G., Embley, T. M., & Goodfellow, M. (1993). Future of bacterial systematics. In: *Handbook of new bacterial systematics* (pp. 513–524). London: Academic Press.
14. Staneck, J. L., & Roberts, G. D. (1974). *Applied Microbiology, 28*, 226–231.
15. Collins, M. D. (1985). *Methods Microbiol, 18*, 329–366.
16. Wu, C., Lu, X., Oin, M., Wang, Y., & Ruan, J. (1989). *Microbiology, 16*, 76–178.
17. Sasser, M. (1990). In Z. Klement, K. Rudolph, & D. Sands (Eds.), *Identification of bacteria through fatty acid analysis in methods in phytobacteriology* (pp. 199–204). Budapest: Akademia Kiado.
18. Kämpfer, P., Kroppenstedt, R. M., & Dott, W. A. (1991). *Journal of General Microbiology, 137*, 1831–1891.
19. Gonzalez, J. M., & Saiz-Jimenez, C. A. (2005). *Extremophiles, 9*, 75–79.
20. Pitcher, D. G., Saunders, N. A., & Owen, R. J. (1989). *Letters in Applied Microbiology, 8*, 151–156.
21. Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt & M. Goodfellow (Eds.), *Nucleic Acid Techniques in Bacterial Systematics* (pp 115–148). Chichester
22. Chun, J., & Goodfellow, M. A. (1995). *International Journal of Systematic Bacteriology, 45*, 240–245.

23. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., et al. (2001). *Lancet, 357*, 1225–1240.
24. Felsenstein, J. PHYLIP (1993). (Phylogenetic Inference Package), version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, USA.
25. Jukes, T. H., & Cantor, C. R. (1969). Evolution of protein molecules. In: H.N. Munro (Ed.) *Mammalian Protein Metabolism* (pp 21–132), New York.
26. Felsenstein, J. (1985). *Evolution, 39*, 783–791.
27. Lechevalier, M. P., Prauser, H., Labeda, D. P., & Ruan, J. S. (1986). *International Journal of Systematic Bacteriology, 36*, 29–37.
28. Dixon, G. H., & Kornberg, H. L. (1959). *Biochem J*, 72–79.
29. Hatona, K., Nishii, T., & Kasai, H. (2003). *International Journal of Systematic and Evolutionary Microbiology, 53*, 1519–1529.
30. Olano, C., Lombo, F., Mendez, C., & Salas, A. (2008). *Metabolic Engineering, 10*, 281–292.
31. Gesheva, V., Ivanova, V., & Gesheva, R. (2005). *Microbiological Research, 160*, 243–248.
32. Cocaign-Bousquet, M., Guyonvarch, A., & Lindley, N. D. (1996). *Applied and Environmental Microbiology, 62*, 429–436.
33. Papagianni, M., & Mattey, M. (2004). *Process Biochemistry, 39*, 1963–1970.
34. Gokarn, R. R., Eiteman, M. A., & Altman, E. (2000). *Applied and Environmental Microbiology, 66*, 1844–1850.
35. Peksel, A., Torres, N. V., Liu, J., Juneau, G., & Kubicek, C. P. (2002). *Applied Microbiology and Biotechnology, 58*, 157–163.
36. Menzel, K., Zeng, A. P., Biebl, H., & Deckwer, W. D. (1996). *Biotechnology and Bioengineering, 52*, 549–560.
37. Henry, L., George, N. B., & San, K. Y. (2005). *Metabolic Engineering, 7*, 116–127.
38. Connett, R. J., & Blum, J. J. (1971). *Biochemistry, 10*, 3299–3309.
39. Wang, Z. X., Bramer, C. O., & Steinbüchel, A. (2003). *FEMS Microbiology Letters, 228*, 63–71.
40. Surowitz, K. G., & Pfister, R. (1985). *Canadian Journal of Microbiology, 31*, 702–706.
41. AyarKayalı, H., & Tarhan, L. (2006). *Enzyme and Microbial Techn, 38*, 727–734.