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Carbohydrate Metabolite Pathways and Antibiotic Production Variations of a Novel *Streptomyces* sp. M3004 Depending on the Concentrations of Carbon Sources

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Abstract To determine the variations of growth, some key enzyme activities such as glucose kinase (GK), glucose-6-phosphate dehydrogenase (G6PDH), α-ketoglutarate dehydrogenase (KGDH), and isocitrate lyase (ICL) besides metabolite levels of pyruvate and antibiotic production of newly isolated *Streptomyces* sp. M3004 were grown in culture media which contain 10–20 g/l concentration with either glucose or glycerol as carbon source. Biomass and intracellular glucose and glycerol levels of *Streptomyces* sp. M3004 showed positive correlation with the concentration of these carbon sources, and these levels were higher in glucose compared with the glycerol-supplemented mediums. GK, G6PDH, and KGDH activities showed marked correlation with the concentration of both glucose and glycerol, and the activity levels were 4.14-, 1.47-, and 1.27-fold higher in glucose than glycerol. A key enzyme of the glyoxalate cycle, ICL activities decreased with increasing glucose concentrations from 10 to 20 g/l, but increased up to 15 g/l of glycerol. The positive correlations were also determined between intracellular glucose and glycerol levels besides pyruvate and protein variations with respect to concentrations of the carbon sources. Antibacterial activities of *Streptomyces* sp. M3004 reached maximum on the stationary phase, while it did not change significantly with respect to glucose and glycerol.

Keywords *Streptomyces* sp. · Carbon sources · Carbohydrate metabolism · Protein · Antibiotic

Introduction

Streptomyces are an important group of the actinomycetes, which are aerobic filamentous bacteria, and have extreme importance in the environment. *Streptomyces*

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are Gram-positive mycelial soil bacteria with high genomic G+C content and a complex life cycle. To identify new isolated *Streptomyces* species, several techniques have been developed, including selective plating methods [1], proof of the presence of L,L-diaminopimelic acid, the absence of characteristic sugars in the cell wall [2], and construction of genetic marker systems [3]. In addition, 16S rRNA sequence data have proven invaluable in *Streptomycetes* systematics, in which they have been used to identify several newly isolated *Streptomycetes* [4]. They have attracted scientific and economic interest because they produce a wide variety of secondary metabolites including antibiotics, antitumoral agents, and insecticides [5, 6].However, little is known about the regulation of carbon utilization and carbohydrate transport, and how these affect secondary metabolism [7]. When bacteria are placed in environments with suboptimal levels of nutrients, they rapidly express appropriate regulatory hunger responses. Studies with carbohydrate-limited chemostats suggest that bacteria induce an array of transport systems to scavenge for substrate with higher affinity and also to broaden its nutritional spectrum [8]. The initial event in the metabolism of carbohydrates is their transport across the cytoplasmic membrane. There are three major uptake systems for sugars in bacteria: (1) the phosphoenolpyruvate (carbohydrate)—phosphotransferase system (PTS), which is involved in both transport and phosphorylation of a sugar at the expense of PEP, resulting in accumulation of the corresponding carbohydrate phosphate (group translocation) [9, 10]; (2) ion-linked sugar transport—secondary transport systems where the sugar uptake is driven by an ion gradient [11]; and (3) carbohydrate transport ATPases—primary transport systems that couple ATP hydrolysis with translocation (ABC transport systems) [12]. In microorganism, carbohydrate is linearly converted to pyruvate through the glycolytic pathway, with production of ATP by substrate level phosphorylation and reducing equivalents (NADH) at the level of glyceraldehyde 3-phosphate dehydrogenase [13, 14]. Although the aerobic metabolic shift was explained on the basis of substrate level regulation, one can infer that altered gene expression in response to aeration can also contribute to the fate of pyruvate. In addition, glucose is also oxidized through the pentose–phosphate pathway (PPP), which is a key component of the cytosolic NADPH regenerating cell machinery. The rate-limiting step in PPP activity is catalyzed by glucose-6-phosphate dehydrogenase (G6PDH), which oxidizes glucose-6-phosphate into 6-phosphogluconate (6-PG), conserving the redox energy as NADPH. The respiratory process continues with the mitochondrial reactions of the TCA cycle, which is a central part of the energetic metabolism that contributes to the generation of ATP by the chemical breakdown of carbohydrates, fats, and proteins.

In this study, the variations of some key enzymes and metabolites of essential carbohydrate pathways such as glucose kinase (GK), α-ketoglutarate dehydrogenase (KGDH), G6PDH, and isocitrate lyase (ICL) activities, as well as pyruvate and antibiotic production, were determined with respect to glucose and glycerol concentrations as carbon sources in growth medium of newly identified *Streptomyces* sp. M3004 isolated from legumen rhizosphere (*Sophora japonica* L.).

Materials and Methods

Streptomyces sp. M3004 was isolated from a legumen *S. japonica* L. rhizosphere and the 16S rRNA gene sequences determined for the *Streptomyces* sp. strain deposited in GenBank database under the accession number AY987376 after the related experimental procedures [15–18].

Media and Growth Conditions

Spore cultures of *Streptomyces* sp. M3004 were prepared by inoculating solid medium M65. This medium contains 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 [19]. The basal chemically defined fermentation medium contained 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 (MOPS) buffer, and 1 ml trace salts solution (containing 1.0 g$FeSO_4$ $7H_2O$, 1 g $MnCl_2$ $4H_2O$, 1.0 g $ZnSO_4$ H_2O, and 1.0 g $CaCl_2$) in 1 l of ultra-pure water. The pH was adjusted to 7.0 before autoclaving. The cultures were inoculated with spore suspensions (OD_{600}=0.15) 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 collected by centrifugation followed by washing twice with distilled water and kept at −20 °C.

Preparation of Cell-Free Extracts

For preparation of cell extracts, wet *Streptomyces* sp. M3004 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 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.

Dry Weight Determinations

Ten-milliliter aliquots of culture were centrifuged, and the pellets were washed and centrifuged twice with deionized water and were placed at 105 °C for approximately 24 h, until the weight remained constant.

Determination of Glucose and Glycerol Levels

Intracellular glucose and glycerol levels were obtained from cells prepared essentially as described by Babul et al. by using a boiling water extraction [20]. The extracted sample was cooled on ice, quickly frozen, and then lyophilized. The lyophilyzate was grown in 1 ml of water and centrifuged twice to eliminate cell debris, and supernatant was filtered through a 0.45-μm filter before injecting into the HPLC.

For analyzing the extracellular metabolites, 1 ml of culture was centrifuged, and the supernatant was then filtered through a 0.45-μm syringe filter for HPLC analysis. The HPLC system used was equipped with Alltech IOA-1000 column, a UV detector, and a differential refractive index detector. Mobile phase, 0.4 ml/min, using 9.0 mM H_2SO_4 solution was applied to the column. The column was operated at 42 °C. Standards were prepared for glucose for both the RI detector and UV detector (210 nm), and calibration curves were created.

Determination of Pyruvate Levels

Pyruvate concentrations were determined with 2,4-dinitrophenylhydrazine [21]. The colored complex formed, which has a maximum absorbance at 520 nm. Samples and

pyruvate solutions containing 0–2.6×10^{-2} mg/ml pyruvate volume up to 3 ml were pipetted into test tubes. One milliliter of 1 mg/ml 2,4-dinitrophenylhydrazine reagent (dissolved in 2 N HCl) was added to the test tube, and the contents were mixed. Absorbance at 520 nm was measured after 10 min. The amounts of pyruvate were plotted against the corresponding absorbance resulting in a standard curve used to determine the pyruvate in unknown samples.

Enzyme Assays

Glucose kinase was assayed by measuring the initial rate of increase in $NADP^+$ absorbance at 365 nm. The reaction mixture contained 100 mM Tris–HCl, pH 7.5, 5.0 mM $MgCl_2$, 0.5 mM $NADP^+$, 10 mM glucose, 1 mM ATP, and 0.7 U G6PDH [22].

Glucose 6-phosphate dehydrogenase activity was obtained by determining the rate of NADPH formation at 340 nm [23].

α-Ketoglutarate dehydrogenase activity was assayed by measuring the initial rate of increase in NAD absorbance at 340 nm. The standard reaction mixture, as optimized during this study, contained 100 mM Tris–hydrochloride buffer (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM MgCl2, 2 mM thiamine PPi, 1 mM sodium α-ketoglutarate, 1 mM NAD^+, 0.2 mM coenzyme A (CoA), and enzyme sufficient to produce an increase in absorbance [24].

Isocitrate lyase was assayed in a mixture for ICL containing, 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 started by the addition of 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 units per milligram of protein, with 1 U enzyme activity equal to 1 nmol glyoxalate formation [25].

Protein Determination

The protein content was determined by the method of Bradford et al. using bovine serum albumin as standard [26].

Disk Diffusion Susceptibility Test

In order to determine antibiotic production, the 3-ml samples, which were taken from the liquid phase of *Streptomyces* sp. M3004 growth in either glucose- or glycerol-supplemented medium, were lyophilized and then resolved with 250 μl distillated water. Twenty microliters of each aliquot samples were pipetted on the overlapped two sterile chromatography paper disks following the National Committee for Clinical Laboratory Standards [27] (NCCLS) recommendation for antimicrobial disk susceptibility tests (Whatman 3Chr, each 6-mm diameter paper disk impregnated with 10 μl aliquot). For the determination of antimicrobial activities of aliquot samples, the following pathogen microorganisms were used: NRRL B-209 *Bacillus subtilis*, ATCC 25922 *Escherichia coli*, NRRL B-1018 *Micrococcus luteus*, ATCC 27853 *Pseudomonas aeruginosa*, ATCC 29213 *Staphylococcus aureus*, ATCC 10231 *Candida albicans*, NRRL 465 *Aspergillus parasiticus*, and isolate NK001 *Aspergillus niger*. In each aliquot impregnated, attached paper disks were placed on the surface of each pathogen microorganism's inoculated plates and then incubated at 37 °C for 24 h. After the incubation period, the diameters of the inhibition zones were measured with a compass.

Statistical Analysis

Tukey test, one of the multiple comparisons, was used for statistical significance analyses. The values are the mean of three separate experiments. Also, comparison was made with *Pearson correlation* for each substrate and/or enzyme depending on glucose concentration with respect to incubation time.

Results

In order to determine the optimal nutritional condition for growth, metabolic cycles such as glycolysis, TCA, PPP, and glyoxalate were used; and for antibiotic production, *Streptomyces* sp. M3004 was grown in culture media which contain either glucose or glycerol as carbon source. The variations of biomass; pH levels of growth medium; GK, GKPDH, KGDH, and ICL activities; and intra/extracellular pyruvate, glucose, and glycerol levels of newly identified *Streptomyces* sp. M3004 isolated from legumen rhizosphere (*S. japonica* L.) were determined with respect to incubation period.

The Variations of Growth Curve and pH Levels of *Streptomyces* sp. M3004 with Respect to Initial Glucose and Glycerol Concentrations

Figure 1a, b indicates that biomass of *Streptomyces* sp. M3004 in both glucose- and glycerol-supplemented medium showed a first stage of rapid growth, and this stage ended approximately 48 h after inoculation. It was followed by a transient stop in growth, a transition phase of about 12 h (48–60 h after incubation) before entering into the stationary phase. In this growth medium, biomass of *Streptomyces* sp. M3004 increased with increasing concentration of carbon sources in the growth medium ($p<0.01$). However, biomass levels in 20 g/L of glucose were 3.25±0.059 at the 48th hour, while it was 2.45±0.038 g/L in 20 g/L of glycerol ($p<0.01$).

As shown in Fig. 2a, pH of *Streptomyces* sp. M3004 growth medium slightly increased in the first 36 h of incubation ($p>0.01$) and then decreased continuingly during the incubation time. These decreases in pH values showed correlation with increases in glucose concentrations. Extracellular pH levels were also increased up to the 36th hour for all glycerol concentrations we tested and then decreased continuingly (Fig. 2b). The pH values

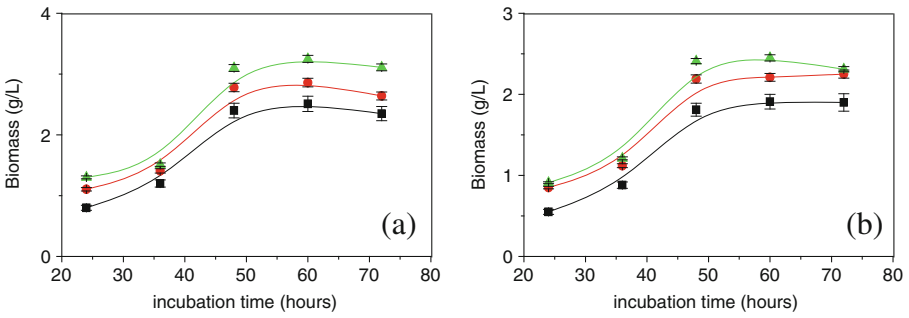

Fig. 1 Variations of biomass glucose (**a**) and glycerol levels (**b**) in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) carbon sources concentrations

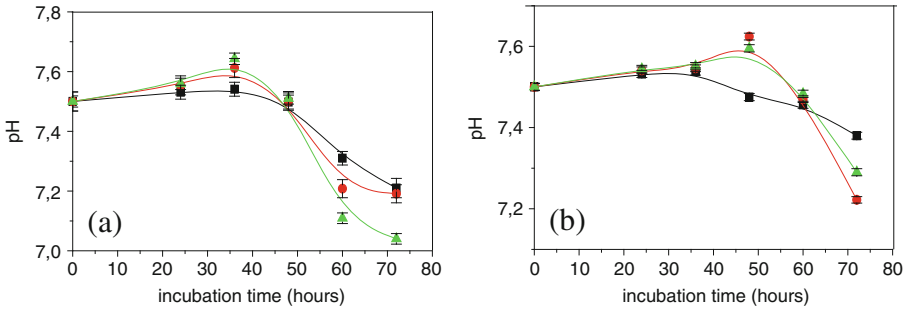

Fig. 2 Variations of pH in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

decreased more at higher glycerol concentrations in the culture medium. The highest decreases in pH levels were 0.46 U for 20 g/l of glucose.

The Variations of Pyruvate and Protein Levels of *Streptomyces* sp. M3004 with Respect to Initial Glucose and Glycerol Concentrations

Figure 3a shows that extracellular glucose levels did not change significantly during the first 36th incubation period and then decreased continuingly for all used glucose concentration ($p<0.01$). Figure 3b shows that extracellular glycerol levels decreased continuingly ($p<0.01$). The rate of glycerol consumption showed a positive correlation with the glycerol concentration of the growth medium. Consumption rates of glucose and glycerol of *Streptomyces* sp. M3004 grown in 20 g/l of both carbon sources were 73.7% and 34.8% at the end of incubation when compared with the initial of incubation.

On the other hand, intracellular glucose levels increased up to the 60th hour of incubation and decreased significantly ($p<0.01$; Fig. 4a). In addition, a positive correlation was determined between intracellular glucose levels and glucose concentration in the growth medium ($p<0.05$). The intracellular glycerol levels increased in the range of 10–20 g/l of glucose in the initial hours of *Streptomyces* 3004 cultivation ($p<0.01$; Fig. 4b) and reached their maximum levels on the 60th hour, after the levels began to decline ($p<0.01$).

As an end product of glycolysis, intracellular pyruvate levels of *Streptomyces* sp. M3004 were increased simultaneously at the very beginning of increases in intracellular glucose

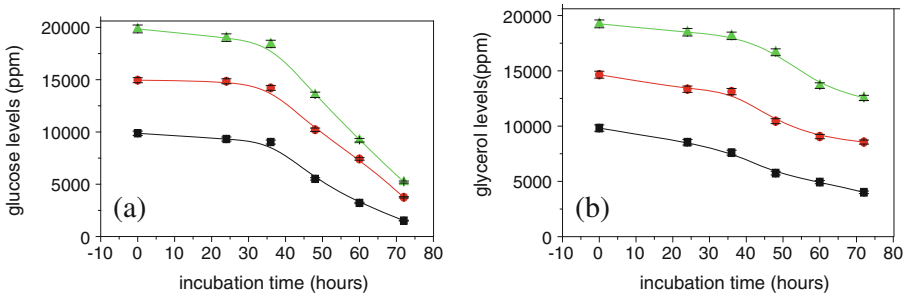

Fig. 3 Variations of residual glucose (**a**) and glycerol levels (**b**) in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) carbon sources concentrations

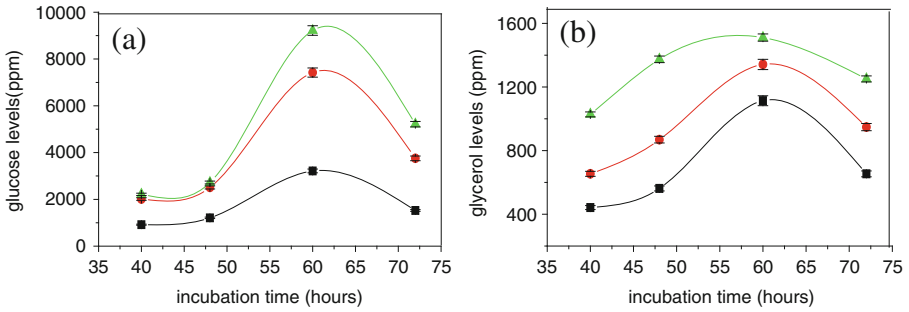

Fig. 4 Variations of intracellular glucose and glycerol levels in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

levels ($p<0.01$; Fig. 5a). Figure 5b shows that very similar intracellular pyruvate concentration variations were obtained when glycerol was substituted with glucose as carbon source in the same period. Intracellular pyruvate levels increased markedly with the increases in concentration of carbon sources used from 10 to 15 g/l, while it did not change at 20 g/l ($p>0.01$). However, extracellular pyruvate levels increased significantly with respect to glucose concentration of the growth medium ($p<0.01$; Fig. 6a). Extracellular pyruvate levels did not change markedly with respect to glycerol concentration of the growth medium ($p>0.01$; Fig. 6b). In addition, extracellular pyruvate levels that were found in glycerol-supplemented culture condition were significantly lower in comparison to glucose ($p<0.01$).

The activities of GK, one of the key enzyme of glycolysis, increased in the range of 10–20 g/l of glucose in the initial hours of *Streptomyces* sp. M3004 cultivation (0.602; $p<0.05$) and reached their maximum levels on the 48th hour after the levels began to decline ($p<0.01$; Fig. 7a). In addition, GK activity also increased with glycerol concentration, while the determined level of 15 g/l glycerol was not lower significantly compared to 20 g/l (Fig. 7b). The maximum activity levels determined in glucose and glycerol were 392.32 and 94.7 IU/mg on the 52nd and 64th hours of incubation, respectively.

G6PDH, the rate-limiting enzyme of PPP, determines the amount of NADPH by controlling the metabolism of glucose via the PPP. As indicated in Fig. 8a, b, the activities of G6PDH showed a positive correlation with the increases in concentration of glucose ($r=0.605$,

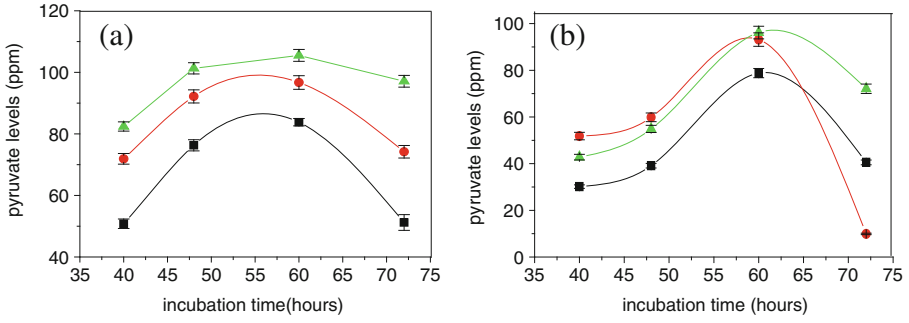

Fig. 5 Variations of intracellular pyruvate levels in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

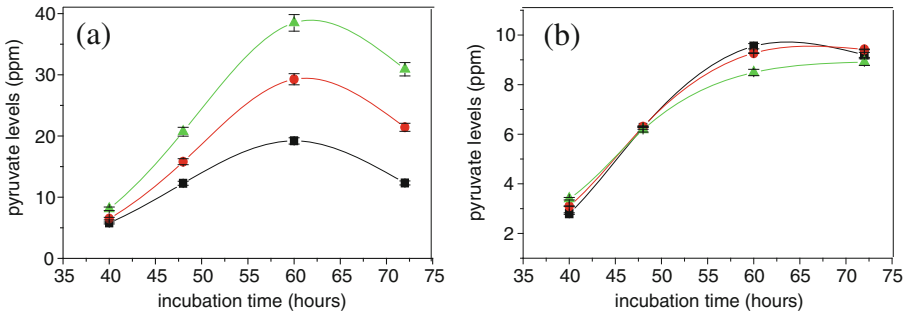

Fig. 6 Variations of extracellular pyruvate levels in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

$p<0.05$), while it increased up to 15 g/l of glycerol. The determined maximum levels of *Streptomyces* sp. M3004 growth in glucose and glycerol on the 52nd and 64th hours were determined as 105.1 and 71.6 IU/mg, respectively.

KGDH enzyme is a mitochondrial enzyme complex that oxidatively decarboxylates α-ketoglutarate to succinyl-CoA in the TCA. According to the results, parallel to the increasing GK activity, the specific activities of KGDH of *Streptomyces* sp. M3004 also increased with increases in glucose and glycerol concentration ($r=0.791$, 0.909; $p<0.01$; Fig. 9a, b). On the other hand, the highest KGDH activities of glucose-supplemented mediums on the 52nd hour were 166.2 IU/mg, while it was 130.4 IU/mg for glycerol on the 64th hour.

The activity of ICL, a key enzyme of the glyoxalate shunt and known to catalyze the reversible cleavage of isocitrate into succinate and glyoxalate, decreased with increasing glucose concentrations from 10 to 20 g/l, but increased up to 15 g/l of glycerol ($p<0.01$; Fig. 10a, b). The variations of the activity of *Streptomyces* sp. M3004 grown in glucose medium had similar trend with glycerol.

As can be seen from Fig. 11a, changes in the protein levels depending on glucose concentration showed similar trends with the intracellular pyruvate levels ($p<0.01$). Upon supplementation with glycerol as an alternative carbon source, protein variations showed the same properties compared to the medium containing glucose (Fig. 11b). However,

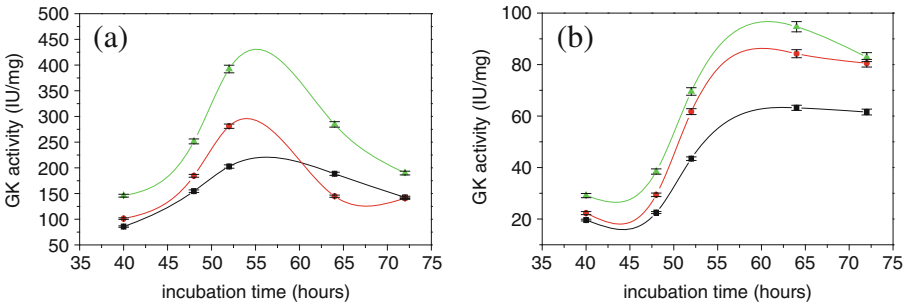

Fig. 7 Variations of GK activity in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

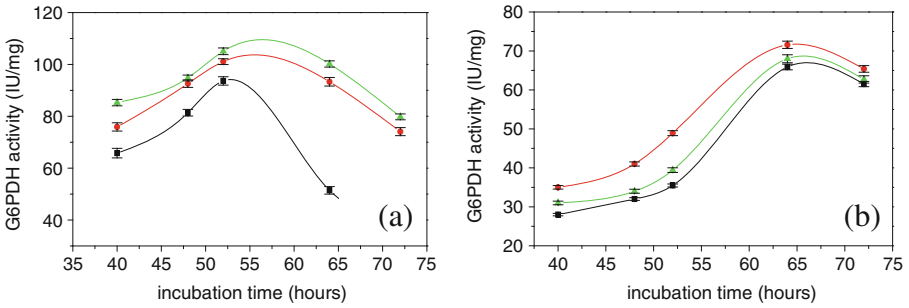

Fig. 8 Variations of G6PDH activity in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

protein levels in glycerol-supplemented medium were comparatively lower than the levels determined in glucose ($p>0.01$).

The Variations of Antibiotic Levels of *Streptomyces* sp. M3004 with Respect to Initial Glucose and Glycerol Concentrations

Antibacterial activities of *Streptomyces* sp. M3004 were assayed for each culture supernatant using the bioassay test against *B. subtilis* (NRRL B-209), *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922), and NRRL B-1018 *M. luteus*; ATCC 27853 *P. aeruginosa*; ATCC 10231 *C. albicans*; NRRL 465 *A. parasiticus*; and *A. niger*. However, antibacterial activities reached maximum on the stationary phase of *Streptomyces* sp. M3004, which was effective only against *B. subtilis* (NRRL B-209), *S. aureus* (ATCC 29213), and *E. coli* (ATCC 25922). The activities of *Streptomyces* sp. M3004 did not change significantly with respect to glucose and glycerol, and their zone diameters were determined as 7.0, 8.8, and 10.3 mm at 10, 15, and 20 g/l of both carbon sources, respectively.

Discussion

A new actinomycete strain named *Streptomyces* sp. M3004 isolated from Turkey soil produced antimicrobial activities against Gram-positive and Gram-negative bacteria [18].

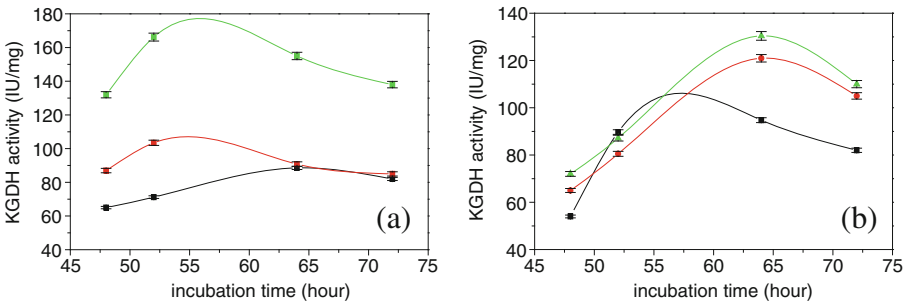

Fig. 9 Variations of KGDH activity in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles* glucose- (**a**) and glycerol-supplemented (**b**) medium

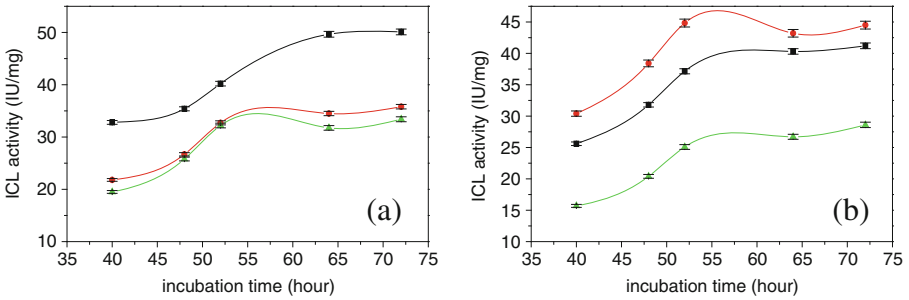

Fig. 10 Variations of ICL activity in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles* and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

Indeed, different *Streptomyces* species produce about 75% of commercially and medically useful antibiotics [28]. Therefore, several studies are oriented towards determination of metabolic precursors related with the antibiotic production from the especially newly isolated *Streptomyces* species.

In this study, biomass of *Streptomyces* sp. M3004 increased with increasing concentration of carbon sources in the growth medium which contained sufficient nitrogen and phosphate concentrations ($p<0.01$). Based on this, it is suggested that increased concentration of glucose and glycerol supported biomass of *Streptomyces* sp. M3004. However, the effect of a switch in carbon utilization from glucose to glycerol was pointed out by an important decrease in the biomass. This result may indicate that glucose is a sole carbon source of the strain. However, a study of various carbon sources in *Streptomyces antibioticus*, including glucose, fructose, and glycerol, concluded that fructose was most effective for production of biomass [29]. Surprisingly, none of the carbon sources classified as pentoses, disaccharides, or trisaccharides were catabolized by *Streptomyces noursei* ATCC 11455 [30]. In contrast to *S. noursei* ATCC 11455, several *Streptomyces* have been demonstrated to grow on galactose, xylose, maltose, sucrose, lactose, and raffinose [31–33]. There is diversity in carbon sources that can be metabolized by different *Streptomyces* species.

According to the results, extracellular glucose and glycerol levels of *Streptomyces* sp. M3004 decreased significantly up to the 48th hour of incubation with corresponding increase in biomass levels. This situation suggests that glucose consumption rate increased

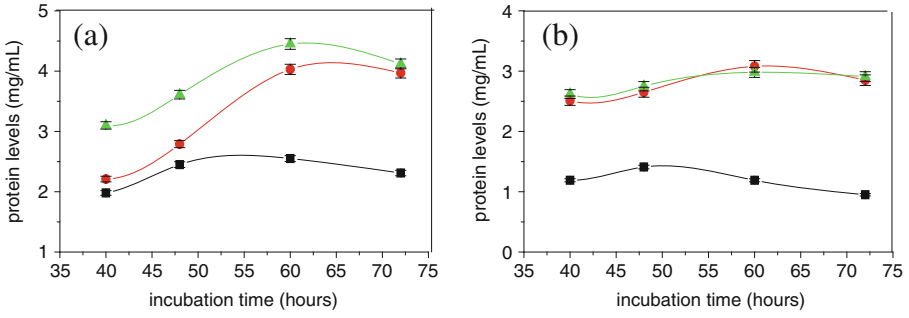

Fig. 11 Variations of protein levels in *Streptomyces* (3004) depending on the incubation period in medium containing 10 (*squares*), 15 (*circles*), and 20 g/l (*triangles*) glucose- (**a**) and glycerol-supplemented (**b**) medium

in order to maintain sporulation of *Streptomyces* sp. M3004. Approximately 10–20% of the initial concentration of glucose added was still present when the cultures were very advanced (72 h). In addition, residual glycerol levels in glycerol-supplemented medium were significantly higher than glucose. The data suggests that glycerol uptake in *Streptomyces* sp. M3004 is scarcely regulated. Determination in higher glucose uptake may indicate that its uptake was simultaneously activated by a protein factor, the characteristics, and mechanism [34].

In the current investigation, the variation of GK activities of *Streptomyces* sp. M3004 with respect to glucose or glycerol concentration of the growth medium increased by showing coherence significantly with the pyruvate production during the log phase of the incubation ($p<0.001$), and then both of these activities and the productions decreased significantly. These results have been interpreted as suggesting that after log phase, decreases in intracellular carbon sources levels were caused to slow down in glycolysis rate, and therefore, pyruvate started to consume ATP production in order to synthesis secondary metabolites [35]. These results also suggested that glycolytic flux or the internal concentration of glucose degradation products play a role in carbon catabolic repression [36]. The determination of higher GK activities and pyruvate production of *Streptomyces* sp. M3004 grown in glucose-supplemented medium compared with glycerol may also suggest that glucose is a sole carbon source for this strain. On the other hand, extracellular pyruvate levels in glycerol-supplemented medium did not change significantly, while they increased with increases in glucose concentration of the growth medium. In addition, extracellular pyruvate levels in glucose-supplemented medium were significantly higher than those of glycerol ($p<0.01$). These results have been interpreted as suggesting that pyruvate was released to the growth medium in order to use carbon source more economically. However, this situation was not determined in glycerol-supplemented medium because of slow uptake of glycerol. Results in this study also showed coherence that pH decreased at higher levels in glucose medium and the extent of decrease being higher at higher glucose concentrations because of the excretion of organic acids such as pyruvate [37].

These changes in the glycolytic enzyme activities and pyruvate concentrations could drive the activation of the PPP as an alternative metabolic pathway to provide substrate. Therefore, the activity of G6PDH was determined as the main rate-limiting enzyme of the PPP. The data showed coherence with the research on the antibiotic methylenomycin in *Streptomyces coelicolor* A3(2), which suggested correlation between carbon flux through the pentose phosphate pathway and production of the antibiotic [38]. The increases in G6PDH activity upon increasing glucose and glycerol concentration and incubation period were accompanied by GK activity. These results suggest that PPP may be activated in response to a demand for energy and nucleic acid biosynthesis, supplying substrate for glycolysis [39].

As a key enzyme of TCA cycle, which is a central part of the energetic metabolism, KGDH activity variation shows correlation with both carbon sources concentration, as was the case also for GK. The marked coherence between these enzyme activities was also observed with respect to incubation period. These results suggest an important role for TCA cycle function in the regulation of glycolysis to provide ATP equivalents for metabolism [40]. The activity of ICL, a key enzyme of the glyoxalate cycle (GLC), decreased with the increases in glucose concentration in contrast to GK, KGDH, and GDPDH activities. These results suggest that the ratio between substrate pathways through TCA and GLC are opposite, to control energy state of cells depending on the availability of substrates in the microorganism [41].

The results also showed that an increase of glucose and glycerol concentration in the *Streptomyces* sp. M3004 culture from 10 to 20 g/l led to 1.74- and 1.95-fold increases in protein production. At the same time, protein levels in glucose-supplemented medium were significantly higher than compared to that found for glycerol ($p<0.01$), as was the case also for biomass, the measured enzyme activities, and pyruvate levels. These results also indicate that glucose is the favored carbon substrate as the major cause of preferential sugar utilization [42].

In conclusion, it can be suggested that the relation between the key enzymes of glycolysis, PPP, TCA, and GLC of *Streptomyces* sp. M3004 was observed with respect to used carbon sources and concentrations. Determination in higher levels of the studied enzyme activities in glucose-supplemented mediums compared to glycerol may be explained by the faster uptake of glucose. GK, G6PDH, and KGDH activities showed a marked correlation with the concentration of both glucose and glycerol. Nevertheless, TCA and GLC are opposite, to achieved adaptation depending on the conditions by induction of glyoxalate cycle as a possible alternative to normal anaplerotic reaction. Antibiotic production of *Streptomyces* sp. M3004 depends on the carbon sources increased with the increases in their concentrations, while the levels of zones were not affected. The results indicated that antibiotic activities showed coherence with the GK, G6PDH activities, as well as biomass and intracellular pyruvate and protein levels. The result is in agreement with the findings of Kreig and Holt [43], who reported that fermentable carbon sources by *Streptomycetes* induced both growth and antibiotic production. The above results also indicated the relationship between the antibiotic production and both glycolysis and pentose phosphate pathways, which are known to be aerobically NADH-, ATP-, and NADPH-generating systems, respectively [44].

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