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Research Article/Araştırma Makalesi

VARIATIONS OF VANCOMYCIN PRODUCTION BY AMYCOLATOPSIS ORIENTALIS DEPENDING ON THE GLUCOSE AND GLYCEROL CONCENTRATIONS AS CARBON SOURCES

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Abstract : Vancomycin production as well as extracellular glucose and glycerol level and pH level of *A.orientalis* growth in different glucose (5-20 g/L) and glycerol (2.5-20 g/L) concentrations of the medium was investigated during the incubation period. *A. orientalis* growth rate increased with the increases in glucose and glycerol concentration while pH levels of the growth medium decreased significantly. In addition, a positive correlation was determined between consumption time of extracellular glucose levels and glucose concentration in the culture medium. Similar results were also determined in glycerol supplemented medium. As a glycopeptid antibiotic, vancomycin production of *A. orientalis* increased with the increases in glucose and glycerol concentrations up to 15 and 10 g/L, respectively. In addition, substitution of glycerol with glucose significantly affected vancomycin antibiotic productions whereas the growth rate was close to that of the each other.

Key word: *A.orientalis*, vancomycin, glucose and glycerol carbon sources

Amycolatopsis Orientalis Besi Ortaminda Karbon Kaynaği Olan Glikoz ve Gliserolün Konsantrasyonuna Bağimli Vankomisin Üretiminin Değişimi

Özet : Değişen glikoz (5-20 g/L) ve gliserol (2.5-20 g/L) derişimlerde büyütülen *A.orientalis* in vankomisin üretim düzeyinin yanısıra ekstrasellüler glukoz, gliserol ve pH düzeyleri inkübasyon periyodu süresince incelenmiştir. *A. orientalis* büyüme hızı artan glikoz ve gliserol derişimleriyle artış gösterirken besi ortamının pH düzeylerinde anlamlı düzeyde azalışlar belirlenmiştir. Bununla birlikte, besi ortamındaki glikoz seviyeleri ile glikozun tüketim zamanı arasında pozitif korelasyon saptanmıştır. Benzer sonuç gliserol içeren ortamda da gözlenmiştir. Glikopeptid antibiyotiklerden biri olan vancomisinin *A. orientalis* de üretimi, besi ortamındaki glikozun 15 g/L ve gliserolün 10 g/L ye kadar artmasıyla anlamlı düzeyde artış göstermiştir. Karbon kaynağı olarak gliserolün yerine glikozun kullanılması, *A. orientalis* in vancomisin üretim düzeyinde anlamlı düzeyde artışa neden olmasına rağmen büyüme hızında etkili olmamıştır.

Anahtar sözcük: A.orientalis, vankomisin , glikoz ve gliserol karbon kaynakları

Introduction

Amycolatopsis are Gram-positive mycelial soil bacteria with high genomic G + C content and a complex life cycle. They have attracted scientific and economic interest because they produce a wide variety of secondary metabolites including antibiotics, antitumoral agents, insecticides and hydrolytic enzymes (Arcamone, 1981; Chater, 1993). However, little is known about regulation of carbon utilization and carbohydrate transport, and how these affect secondary metabolic production such as antibiotic. It has been shown that changes in environmental conditions influence the secondary metabolite such as antibiotic production. Although many microorganisms are able to use a variety of different carbon and energy substrates and adapt their enzymatic equipment, their metabolism specifically to the availability of a given substrate or substrate mixture. In addition, variation of temperature, pH-value or dissolved oxygen tension leads to drastic changes in specific growth rate and product concentrations ((Prosser and Tough, 1991). The variation of the growth rate itself in chemostate culture under carbon limitation leads to a different product pattern, as well (Melzoch et al., 1997) because some carbon sources may incorporate as precursors or their amino groups transfer to specific intermediate products (Omura and Tanaka, 1986; Doull and Vining, 1990; Cheng et al., 1995). Therefore, optimization of antibiotic production in actinomycetes strains is the one of the main objective of investigations on the influence of cultivation conditions (Spizek and Tichy, 1995). As an important actinomycetes strain, *Amycolatopsis orientalis* strain produces the glycopeptide vancomycin antibiotic which is effective on the MRSA infections which are extremely difficult to treat because they are also multiple-antibiotic resistant.

In this study, the correlations between extracellular glucose and glycerol levels variations and growth curve, extracellular pH levels and vancomycin productions of *A. orientalis* growth in different glucose and glycerol concentrations were investigated during the incubation period.

Material and Method

Amycolatopsis orientalis was obtained from DSMZ collection of microorganisms. Spore cultures of *Amycolatopsis orientalis* 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, 20 g starch in 1 litre of ultra-pure water (Lechevalier, 1986). 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$FeSO_4$ $7H_2O$, 1g $MnCl_2$ $4H_2O$, 1.0 g $ZnSO_4$ H_2O, 1.0 g $CaCl_2$) in 1 litre 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 hours. After the cultivation process, the cells were collected by centrifugation followed by washing twice with distilled water and kept at -20°C.

Ten-milliliter aliquots of culture were centrifuged and the pellets were washed and centrifuged twice with deionized water and the placed at 105 ^{0}C for approximately 24 h, until the weight remained constant. Optical density was measured at 620 nm with a spectrophotometer.

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.

Vancomycin in culture filtrates was recovered by solid phase extractions using Varian Bond Elut LRC C18 EWP cartridges (Bauchet, 1987). The antibiotic concentration was measured by high-performance liquid chromatography (HPLC) gradient and singles multiple wavelength detection at 210 nm (Backes, 1998) by using Varian Cromsep C8 column.

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 glucose concentration with respect to incubation time.

Results

In this study; growth curve, the variations of pH, extracellular glucose and glycerol levels and vancomycin levels of *Amycolatopsis orientalis* growth in various glucose (5-20 g/L) and glycerol (2.5-20 g/L) concentrations were investigated during the incubation period as the fundamental parameters.

As shown in Figure 1, *A. orientalis* displays logarithmic growth in growth medium during the first 24-36 hour for 5 g/L glucose and 24-48 hour in the range of 10-20 g/L glucose, after which it enters a stationary phase. *A. orientalis* growth rate increased when the concentration of glucose was increased up to 15 g/L. From this concentration and above, growth rate did not increase significantly indicating that glucose was no longer a growth limiting factor for final biomass.

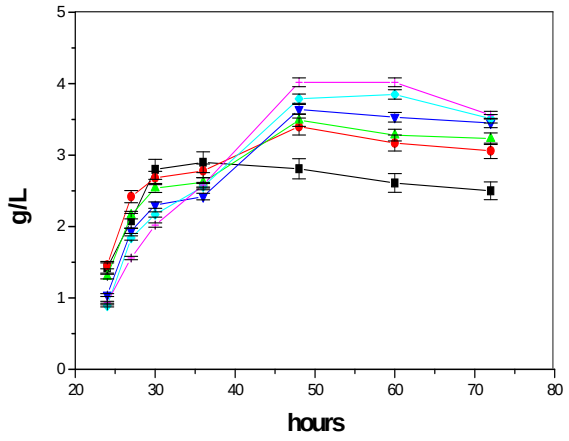

Figure 1. Biomass concentration variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments

Figure 2 shows that logarithmic growth of *A. orientalis* were continued to 36th hour in the range of 2.5 -10 g/L glycerol and 48th hours at 15,20 g/L glycerol. In addition, *A. orientalis* growth rate increased when the concentration of glycerol was increased.

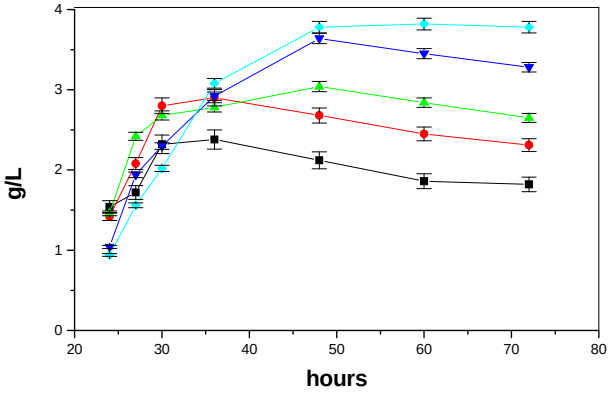

Figure 2. Biomass concentration variations in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

According to our results, pH level of *A. orientalis* showed a rise up to 36th hour for all used glucose concentration and then decreased continuingly until 48th for 5-12.5 g/L, 60th for 15.0 g/L and 72nd hour for 17.5 and 20.0 g/L glucose (Figure 3). These decreases in pH values were increased with increases in glucose concentrations in the culture medium. The highest pH decrease was determined at 20 g/L glucose as 1.04 units. On the other hand, in the following incubation periods, pH variations were slightly increased.

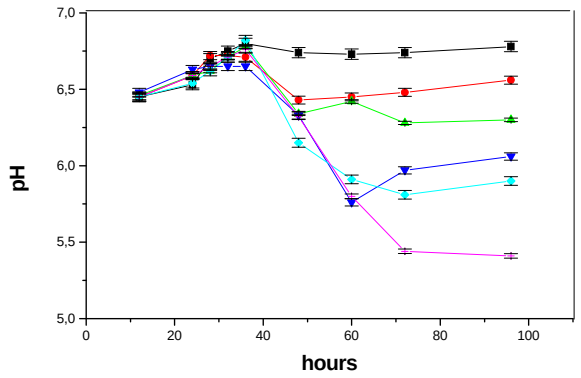

Figure 3. Variations of pH values in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments

As shown in Figure 4, pH level of *A. orientalis* showed a rise up to 28 for 5 and 10 g /L glycerol and up to 32th hour and then decreased continuingly until 36th for 5-12.5 g/L, 60th for 15.0 g/L and 72nd hour for 17.5 and 20.0 g/L glycerol. These decreases in pH values were increased with increases in glycerol concentrations in the culture medium. The highest pH decrease was determined at 20 g/L glycerol. On the other hand, in the following incubation periods, pH variations were slightly increased.

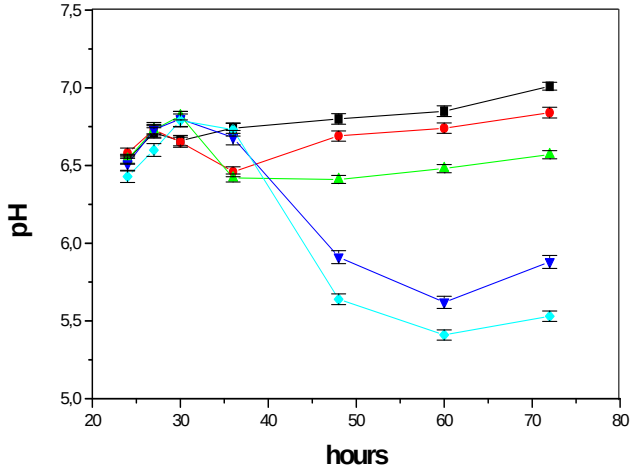

Figure 4. pH variations in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

As shown in Figure 5, a positive correlation was determined between consumption time of extracellular glucose levels and glucose concentration in the culture medium ($r = 0.618$, $p<0.01$). According to the results, glucose in the culture medium was consumed rapidly in the same time period of incubation as stationary phase was observed.

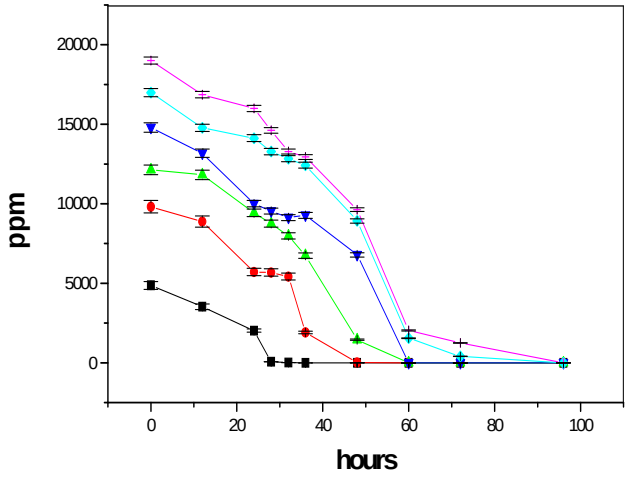

Figure 5. Variations of extracellular glucose level in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments

The results showed that the rate of glycerol transport was increased with respect to incubation time, but it didn't change dependent on the glycerol concentration of the *A. orientalis* growth medium (Figure 6).

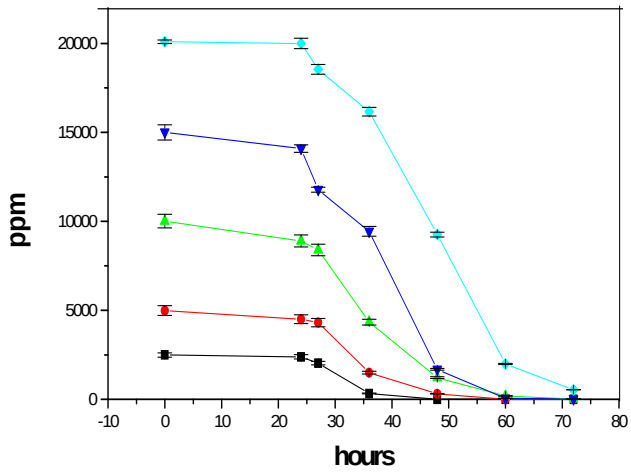

Figure 6 Variations of extracellular glycerol level in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

According to our results, vancomycin productions were reached their maximum values on the 48th hour for all used glucose concentrations except for 5 g/L glucose and afterward, all of them decreased slightly ($p>0.01$) (Figure 7). Vancomycin production increased with the increases in glucose concentrations up to 15 g/L and determined as 7.8 ± 0.086 ppm .

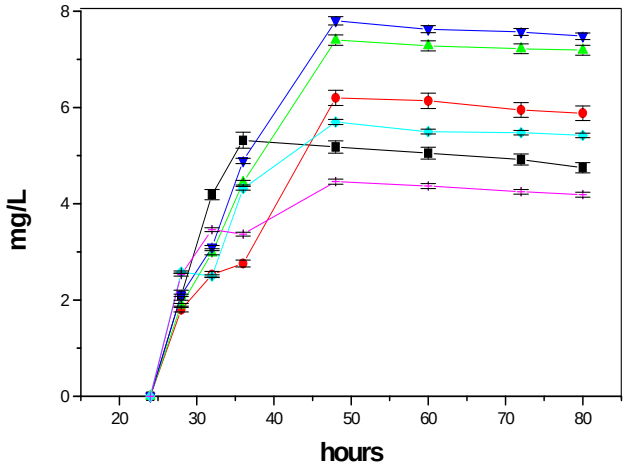

Figure 7. Vancomycin production variations in *A. orientalis* depending on the incubation period in medium containing; 5g/l (—■—), 10g/l (—●—), 12.5g/l (—▲—), 15 g/l (—▼—), 17.5g/l (—♦—), 20g/l (—┼—) glucose. The values are the mean ±SEM for experiments of three separate experiments

As shown in Figure 8, vancomycin productions were reached their maximum values on the 48th hour for all used glycerol concentrations and they decreased for the following incubation period. Vancomycin production increased with the increases in glycerol concentration from 5 to 10 g/L and it decreased significantly at the higher concentration of the glycerol. The maximum vancomycin level was determined as 4.01 ± 0.06 ppm at the 10g/L glycerol.

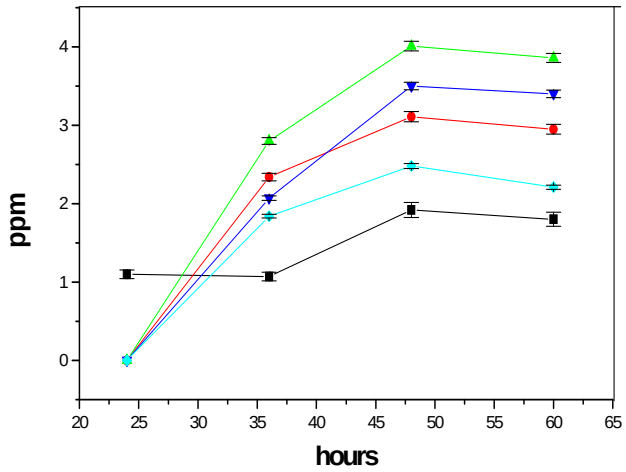

Figure 8 Vancomycin production variations in *A. orientalis* depending on the incubation period in medium containing; 2.5g/l (—■—), 5.0 g/l (—●—), 10g/l (—▲—), 15 g/l (—▼—), 20 g/l (—♦—) glycerol. The values are the mean ±SEM for experiments of three separate experiments

Discussion

Species of the genus Actinomyceletes are filamentous soil bacteria that have long been identified as sources of antibiotics. They are used in large scale productions to supply over 50% of the antimicrobial compounds used routinely in medicine and veterinary medicine. In addition, nature and concentration of some components in fermentation medium have a marked effect on antibiotic production (Doull and Vining, 1990; Lebrihi et al., 1992; Cheng et al., 1995; Coisne et al., 1999; Sanchez and Demain, 2002). When carbon or nitrogen source is a limiting factor, growth is rapidly reduced and antibiotic biosynthesis takes place in the stationary phase (Lee et al., 1997; Parekh et al., 2000). In other cases, antibiotic production is associated with the growth phase. Therefore, special attention has to be paid to the medium composition.

In this study, *A. orientalis* growth rate increased when the concentration of glucose and glycerol was increased up to 15 g/L. From this concentration and above, growth rate did not increase significantly indicating that carbon sources were no longer a growth limiting factor for final biomass (Jonsbu E et al., 2002). In addition, biomass of *A. orientalis* growth in both glycerol and glucose supplemented medium was increased rapidly up to 36th hour and decreases in increases rate were determined in the range of 36th-48 th hour incubation period. These decreases rate were more in glycerol medium compared to glucose. The determined highest biomass didn't change significantly in glucose and glycerol supplemented medium.

According to the results, rapid consumption of glucose in the growth medium showed coherence with the significant increases of the glucose concentration of the growth medium on the 24th hour. In addition, the decreases in glycerol levels were so slow for the first 24 h incubation period and they increased with respect to glycerol concentration for the 28-48 hours. This rapid uptake of glucose compared to glycerol may be because of facilitated and rapid diffusion process of glucose as well as carbon catabolic repression.

pH levels of *A. orientalis* growth in both glycerol and glucose supplemented medium were similar up to 36th hour then decreased continuingly for following incubation period. These decreases in pH values of the *A. orientalis* growth medium were increased with increases in glucose and glycerol concentrations in the culture medium possibly because of excretion of organic acids such as TCA and anaerobic metabolites. Also some researchers showed that intracellular pH was maintained at constant level by leading to the excretion of some organic acid which is profoundly dependent on the nature of the growth limitation and physiology of species (Maharjan, 2005). In addition, slightly increases in pH levels with respect to aging period may shows back uptake of organic acids because of the decreases in carbon sources.

According to the results, increasing the concentration of carbon sources at the optimum concentrations results in a larger production of vancomycin. In addition, much higher vancomycin productivity were obtained in glucose supplemented medium compared to the glycerol has. Also, the use of glucose as a carbon sources promoted a higher production that found in the literature (Dunstan, 2000). Finally, different concentrations of glucose are effective on the vancomycin production yields as well as growth curve and pH levels of the growth medium.

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