Optimal Conditions for the Production of Kanamycin by *Streptomyces kanamyceticus* Mutants

Onanong Pringsulaka[1] and Surina Chavanich[1]

Productions of kanamycin by 3 mutants of *Streptomyces kanamyceticus* K1 were investigated. The mutant UUNNK1 capable of producing the highest amounts of kanamycin was selected. Optimal conditions for kanamycin production in shaking cultures were found when the UUNNK1 was grown in a KPMB medium consisting of (g/l): starch, 15; soytone, 8; bacto-peptone, 1; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 1; $CaCO_3$, 5; NaCl, 3; KCl, 0.5; pH 8.0-8.6, at 30 ^{0}C on a rotary shaker. Under these conditions, within 3 days, the UUNNK1 produced 200 μg/ml of kanamycin which was 14-fold higher than the amount produced by the original strain K1. The production of kanamycin in a 5-litre fermentor was also investigated. Using similar conditions of a shaking flask culture, with agitation speed of 300 rpm, aeration rate at 1.3 vvm, without pH control, UUNNK1 produced the highest amounts, 350 μ g/ml, of kanamycin in 4 days of cultivation. This was 25-fold higher than the yield produced by K1.

Key words: *Streptomyces kanamyceticus,* optimal condition**,** production, kanamycin, mutants.

[1] *Department of Microbiology , Faculty of Science , Chulalongkorn University, Bangkok 10330, Thailand.*

ภาวะที่เหมาะสมต่อการผลิตคานามัยซินโดยสายพันธุ์กลายของ *Streptomyces kanamyceticus*

อรอนงค์ พริ้งศุลกะ และสุรีนา ชวนิชย์ (2542)
วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **24 (2)**

จากการผลิตคานามัยซินโดยสายพันธุ์กลายของ *Streptomyces kanamyceticus* K1 จำนวน 3 สายพันธุ์ พบว่าสายพันธุ์กลาย UUNNK1 สามารถผลิตคานามัยซินได้สูงสุด ดังนั้นจึงนำสายพันธุ์ดังกล่าวมาเลี้ยงในอาหารเหลวเคพีเอ็มบี พบว่าภาวะที่เหมาะสมต่อการผลิตคานามัยซินของสายพันธุ์ UUNNK1 ในระดับขวดเขย่า คืออาหารเลี้ยงเชื้อที่ประกอบด้วย แป้ง 15 กรัม ซอยโทน 8 กรัม แบคโต-เปปโทน 1 กรัม $MgSO_4 \cdot 7H_2O$ 0.5 กรัม K_2HPO_4 1 กรัม $CaCO_3$ 5 กรัม NaCl 3 กรัม และ KCl 0.5 กรัม ในอาหาร 1 ลิตร โดยปรับค่าความเป็นกรด-ด่างเริ่มต้นที่ 8.0-8.6 ณ อุณหภูมิ 30 องศาเซลเซียส บนเครื่องเขย่าชนิดโรตารี ด้วยภาวะการเพาะเลี้ยงดังกล่าว สายพันธุ์ UUNNK1 สามารถผลิตคานามัยซินได้ 200 ไมโครกรัมต่อมิลลิลิตร ในเวลา 3 วัน เมื่อเปรียบเทียบการเลี้ยงสายพันธุ์ UUNNK1 ในสูตรอาหารเดิมที่ใช้กับสายพันธุ์ตั้งต้น K1 แล้ว สายพันธุ์ UUNNK1 สามารถผลิตคานามัยซินได้มากกว่าสายพันธุ์ตั้งต้นถึง 14 เท่า จากการทดลองการผลิตคานามัยซินโดยสายพันธุ์กลาย UUNNK1 ในถังหมักขนาด 5 ลิตร ใช้ภาวะเดียวกับระดับขวดเขย่า โดยให้อัตราการกวนเป็น 300 รอบต่อนาที อัตราการให้อากาศเท่ากับ 1.3 ลิตรต่อ 1 ลิตรของอาหารต่อนาที โดยไม่ควบคุมค่าความเป็นกรด-ด่าง พบว่าสายพันธุ์ UUNNK1 สามารถผลิตคานามัยซินได้สูงถึง 350 ไมโครกรัมต่อมิลลิลิตร ในเวลา 4 วัน ซึ่งสูงกว่าสายพันธุ์ K1 25 เท่า

คำสำคัญ *Streptomyces kanamyceticus*, ภาวะที่เหมาะสม, การผลิต, คานามัยซิน, สายพันธุ์กลาย

INTRODUCTION

Kanamycin is an aminoglycoside antibiotic, produced by *Streptomyces kanamyceticus*. It was discovered by Umezawa and co-workers in 1957.[1] Kanamycin is a water-soluble basic antibiotic and is active against Gram positive, Gram negative and acid-fast bacteria.[2] At present, kanamycin is one of the most important antibiotics applied to human and animal therapy. Its use is widespread and seems to be increasing annually. There are no reports of industrial production of kanamycin in Thailand in spite of its high demand in the Thai market. This is due to the shortage of a potential strain used for antibiotic production. Since improvement of a strain is an effective means to increase the production, therefore in a previous study by our group,[3] we improved *S. kanamyceticus* K1 to increase kanamycin production by mutation. Three mutants designated UUNK15, UUNNK1 and UUNNK25 were obtained. Two mutants produced higher amounts of kanamycin than the parental strain K1 did. The morphology and certain characteristics of the mutants were obviously different from K1, so our initial work involved selection of a stable mutant capable of producing the highest yields of kanamycin.

In this paper, we describe optimal conditions for kanamycin production by UUNNK1 in a shaking flask culture, including the composition of the production medium KPMB, temperature and pH for the production. In addition, the production of kanamycin in a 5-litre jar fermentor was also reported.

MATERIALS AND METHODS

Microorganisms

Streptomyces kanamyceticus K1 was obtained from the Laboratory of Applied Microbiology, Kyushu University, Japan.

Staphylococcus aureus ATCC6538P was used as a testing organism for kanamycin activity.

Mutant strains UUNK15, UUNNK1 and UUNNK25 were developed in our laboratory.[3]

Strain Maintenance

S. kanamyceticus was maintained on YS agar[4] containing (g/l): starch, 10; yeast extract, 3; agar, 15; 1000 ml distilled water, incubated at room temperature for 7-10 days to obtain good growth, then stored at 4°C prior to use.

S. aureus was grown on M1 medium[5] containing (g/l): peptone, 6; casein, 4; yeast extract, 3; beef extract, 1.5; dextrose, 1; agar, 15 at 37°C overnight.

Preparation of Seed for Cultivation and Kanamycin Production

Spores seven to ten days old of *S. kanamyceticus* on YS agar slant were washed by suspending in 0.01% Tween 80. The suspension was filtered aseptically. The number of spores was determined by a haemacytometer and diluted to approximately 10^6 spores per ml.

A one-millilitre spore suspension of *S. kanamyceticus* was added to 50 ml of GPY medium[6] containing (g/l): glucose, 10; bacto-peptone, 4; yeast extract, 4; K_2HPO_4, 4; KH_2PO_4, 2 in a 250 ml Erlenmeyer flask for cultivation on a rotary shaker at 200 rpm at room temperature for 48 hrs. Then 10 ml. of culture medium was transferred to 50 ml. KPMB medium[7] contained (g/l): starch, 20; soytone, 12; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 1; NaCl, 3; bacto-peptone, 3; $CaCO_3$, 5 in a 250 ml. Erlenmeyer flask for 7 days of incubation under conditions as mentioned above.

Determination of Kanamycin Production by Microbiological Assay.[8]

Ten millilitres of sterile normal saline were added to culture slant of *S. aureus* on a M1 medium. Cell suspension was measured at 530 nm absorbance and was diluted to a 0.1 absorbance value.

During 7 days of fermentation, 5 ml samples of production medium were tested for kanamycin activity against *S. aureus* as a test organism. The estimation of kanamycin yields (μg/ml) was measured by microbiological

assay.[8] Standard kanamycin was used to quantify the amounts of antibiotic produced.

Quantitative Analysis by HPLC[9]

Twenty microlitres of culture filtrate from the production medium and different concentrations of kanamycin A sulfate standard were detected by HPLC with a refractive index (RI) detector. Separation of the antibiotic was made by column Lichrocarp C_{18} reverse phase. The mobile phase solvent was 0.02 M potassium phosphate buffer pH 7.5, acetonitrile and methanol in the ratio 40:45:15. The flow rate was 1.5 ml /min and the temperature was 45 °C.

Determination of Cell Dry Weight, Reducing Sugar, Total Sugar and Protein

During 7 days of cultivation, the culture broth as sampled every 24 hrs. The mycelial dry weight and pH of the culture were determined. The amounts of reducing sugar, total sugar and protein were measured by DNSA,[10] phenol sulfuric[11] and Lowry methods,[12] respectively.

Selection of Mutant Strains Producing a Higher Amount of Kanamycin

Ten millilitres of each seed medium of *S. kanamyceticus* K1 and the mutants were transferred in 50 ml KPMB medium for kanamycin production. After 7 days of cultivation, the cell dry weight, amounts of reducing sugar and total sugar, and protein, pH and amount of kanamycin were determined. Selected strains producing the highest amounts of kanamycin was used for studying optimal conditions in shake-flask cultures.

Time Course for Measuring the Growth Rate of Selected Mutant Strains

To study the time course of the selected mutant strain in order to obtain the maximal growth rate of seed culture in GPY medium, the culture broth was taken every 6 hrs for 120 hrs of cultivation. The broth was determined for the mycelial dry weight, pH and reducing sugar.

Selection of C-Source Utilized in Kanamycin Production

Mycelia of the selected mutants were added to KPMB medium in Erlenmeyer flasks with one of the following different C-sources (20 g/l): starch, maltose, lactose, glucose, galactose or sucrose.

The selected C-source, in different amounts of 5-30 g/l, was added to each KPMB medium for kanamycin production.

Selection of N-Source Utilized in Kanamycin Production

Mycelia of the selected mutants were added to Erlenmeyer flasks containing KPMB medium, with proper amounts of the C-source and various N-sources (using a quantity of nitrogen equivalent to the amount of nitrogen in soytone) of 12 g/l: alanine, glucosamine, sodium nitrate, soytone, yeast extract or soybean hydrolysate.

Then, different amounts of selected N-sources (6, 8, 10, 12 and 14) g/l were added to KPMB medium for kanamycin production.

Detection of pH and Temperature for Kanamycin Production

The selected mutants were grown in KPMB medium with various initial pH values ranging from 7.0-8.6 and different temperatures: 28, 30, 35 and room temperature.

Determination of Kanamycin Production in a 5 L Fermentor

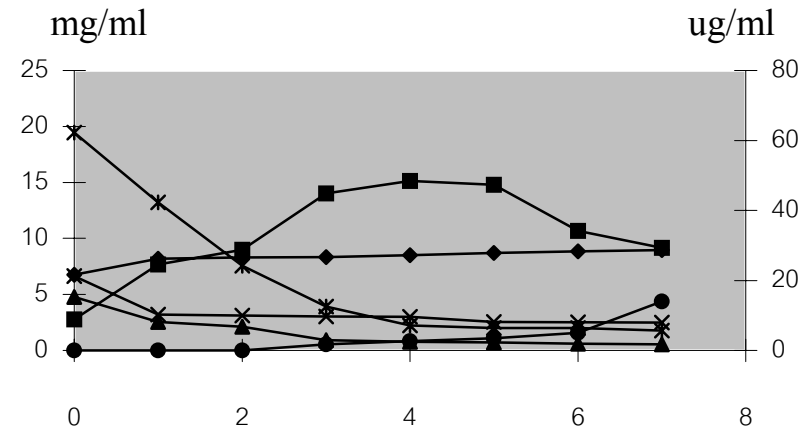
Two hundred and fifty millilitres of GPY medium of the cultivated mutant was transferred to 2250 ml of KPMB medium in a 5 L fermentor with various agitation speeds of 200, 300 and 400 rpm with an aeration rate at 1.3 vvm.

RESULTS AND DISCUSSION

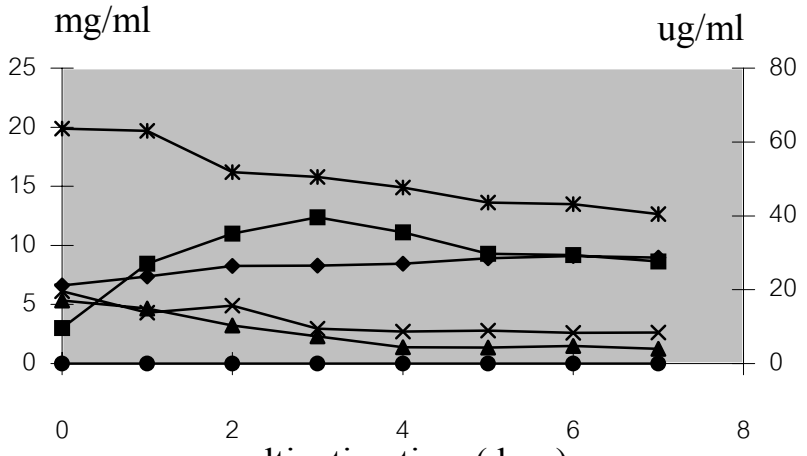
Comparison of Mutant Strains Production of Kanamycin

The K1 and three mutant strains, UUNK15, UUNNK1 and UUNNK25, were cultivated in KPMB medium for kanamycin production. After 7 days of cultivation, cell dry weight, amount of reducing sugar, total sugar, protein, pH and kanamycin concentration were determined, as shown in Fig. 1.

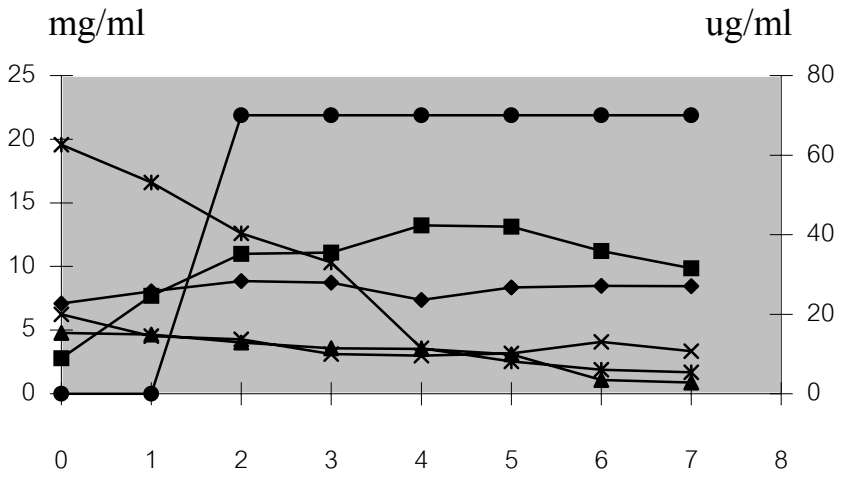
Comparing between parental and mutant strains, cell dry weight and pH of the parental and mutant strains increased after 1 day of cultivation. Amounts of kanamycin, 70 μg/ml and 32 μg/ml, produced by UUNNK1 and UUNNK25, respectively, were higher than that produced by K1. It was noticable that the amount of kanamycin produced by UUNK15 was undetectable. This might be due to losing kanamycin producing activity. Therefore, UUNNK1 was selected for our study. The UUNNK1 strain was not only giving maximal yields of kanamycin but also was stable in producing kanamycin in each experiment. Its yield was 5-folds higher than that produced by K1 (Fig. 1).



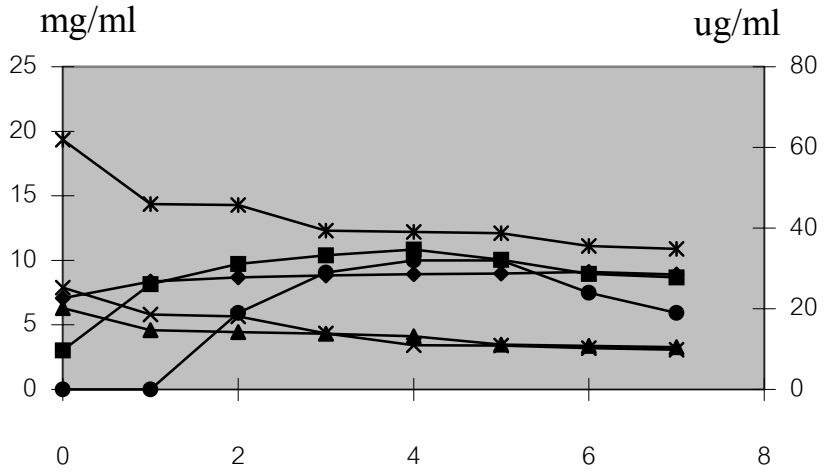
(a)



(b)



(c)



(d)

Figure 1. Determination of cell dry weight, amounts of reducing sugar, total sugar, protein, pH and kanamycin concentration of K1 (a), UUNK15 (b), UUNNK1 (c) and UUNNK25 (d) (■ = cell dry weight, ▲= reducing sugar, ✶ = total sugar, ✖ = protein, ◆ = pH, ● = kanamycin).

Time Course for the Growth Rate of Selected Mutant Strain.

Time course for obtaining the highest growth rate in GPY medium which used as seed medium of UUNNK1. It was found that the maximal dry weight for growth was 2.84 mg/ml at 48 hours; therefore, UUNNK1 was cultured in GPY medium for 48 hours before transferring to production medium of KPMB.

C-Source Utilization in Kanamycin Production.

Various C-compounds were tested as C-sources for the production of kanamycin. As shown in Fig. 2, starch was the best C-source compared with other tested compounds. When different amounts of starch (g/l), 5, 10, 15, 20, 25 and 30, were added to the production medium, the kanamycin yields were 29, 128, 160, 120, 120, 120 respectively. The maximal yield of kanamycin was obtained with 15 g/l of starch. This result was in accordance with the result reported by Umezawa et al. in 1957[1], who reported that starch concentration as giving highest yields of kanamycin.

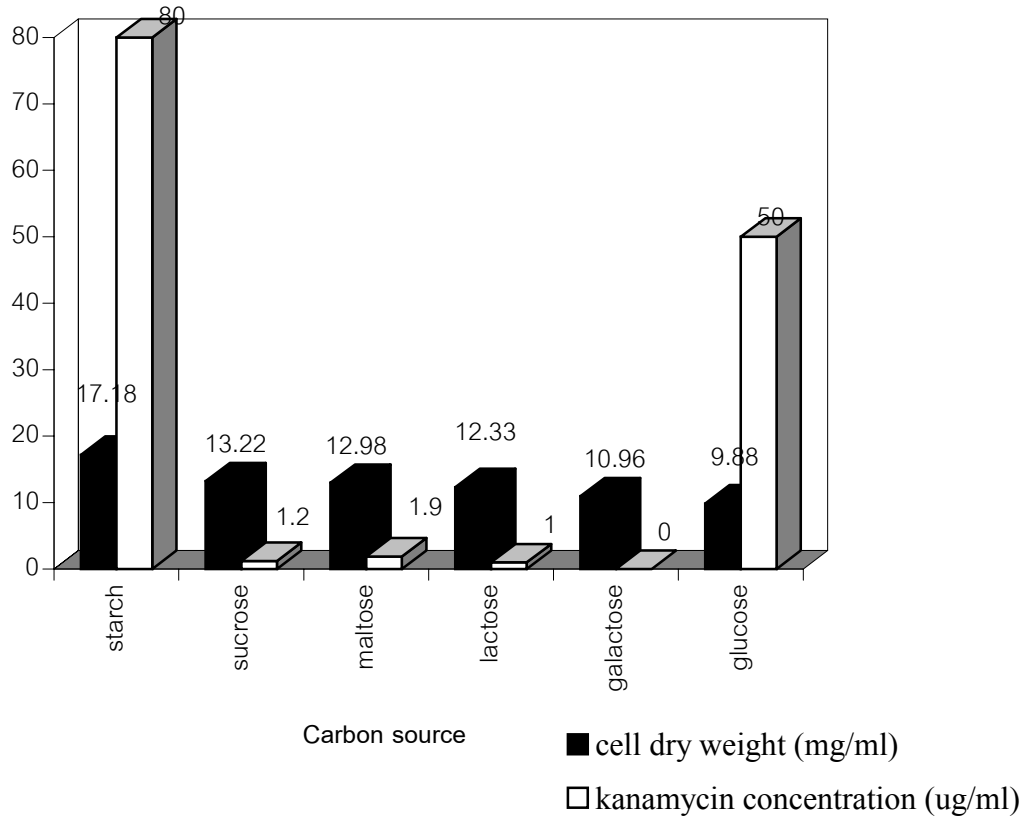


Figure 2. Effect of C-source on kanamycin concentration and cell dry weight.

The pH and Temperature Optima for Kanamycin Production

As shown in Fig. 3 and 4, when using 15 g/l starch as the proper amount of C-sources for a KPMB medium, the optimal pH for maximal yields of kanamycin ranged from 8.0-8.6. Our results were consistent with Umezawa et al.[1] and Basak and Majumdar[14] who reported that kanamycin could be produced when the pH of the medium was higher than 8.0 and the optimal temperature was 30°C. If the temperature was increased to above 35°C, kanamycin would not be obtained. Therefore we controlled the temperature to be below 30°C. This result

indicated that UUNNK1 was sensitive to a higher temperature, in accordance with Umezawa et al. who reported that *S. kanamyceticus* K-2J could produce maximal yields of kanamycin when the temperature ranged from 28 to 32°C.

N-Source Utilization in Kanamycin Production

When various N-sources were tested for kanamycin production. It was found that soytone gave the highest yields of kanamycin, while sodium nitrate, yeast extract and soybean hydrolysate gave moderate yields. Glucosamine gave lower yields and alanine gave undetectable yields (Fig. 5).

With different amounts of soytone of 6, 8, 10, 12 and 14 g/l added to the production medium of KPMB, the kanamycin production and cell dry weight were determined. Highest yields of kanamycin obtained when using 8 and 10 g/l of soytone, whereas 6, 12 and 14 g/l soytone gave moderate yields of 160 μg/ml kanamycin.

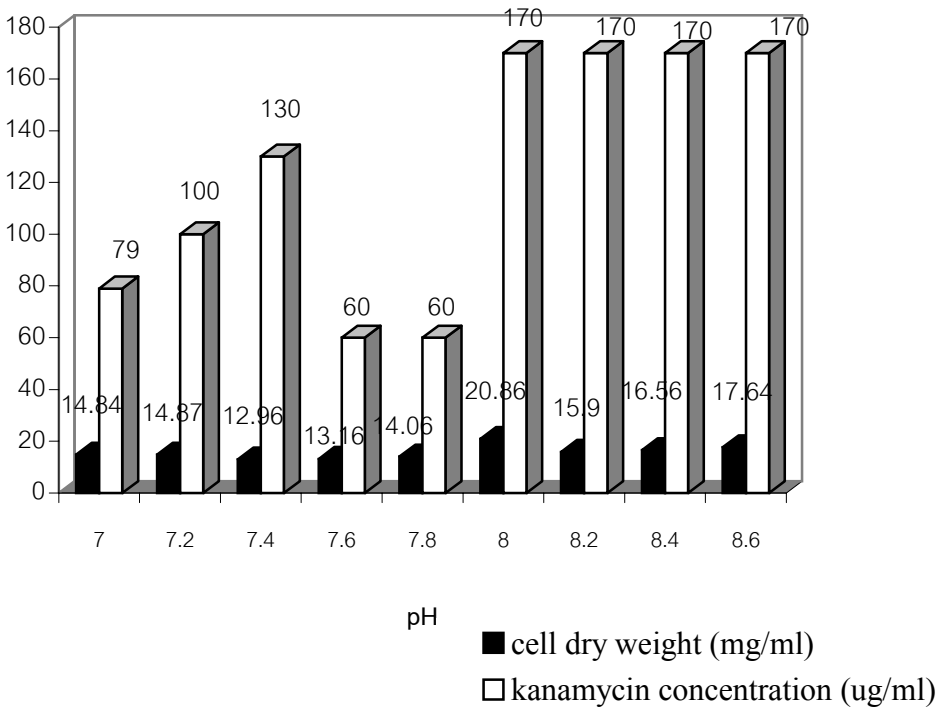


Figure 3. Effect of pH on kanamycin concentration and cell dry weight.

Determination of Kanamycin Production in a 5 L Fermentor

The selected mutant was initially grown in GPY medium and then transferred to KPMB medium in a 5 L fermentor with the same conditions as in the flask culture using different agitation speeds of 200, 300 and 400 rpm. It was found that at 300 rpm, the maximal yields of kanamycin was 350 μg/ml while at 200 rpm or 400 rpm the yield was 70 or 60 μg/ml, respectively.

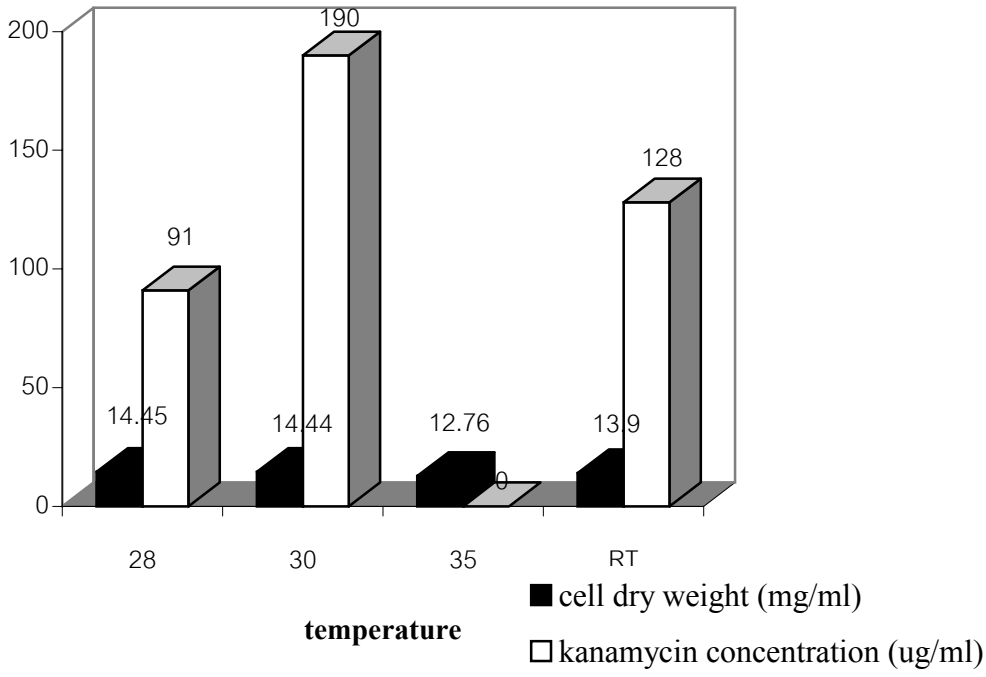


Figure 4. Effect of temperature (°C) on kanamycin concentration and cell dry weight

(RT = Room temperature: 30 ± 3°C)

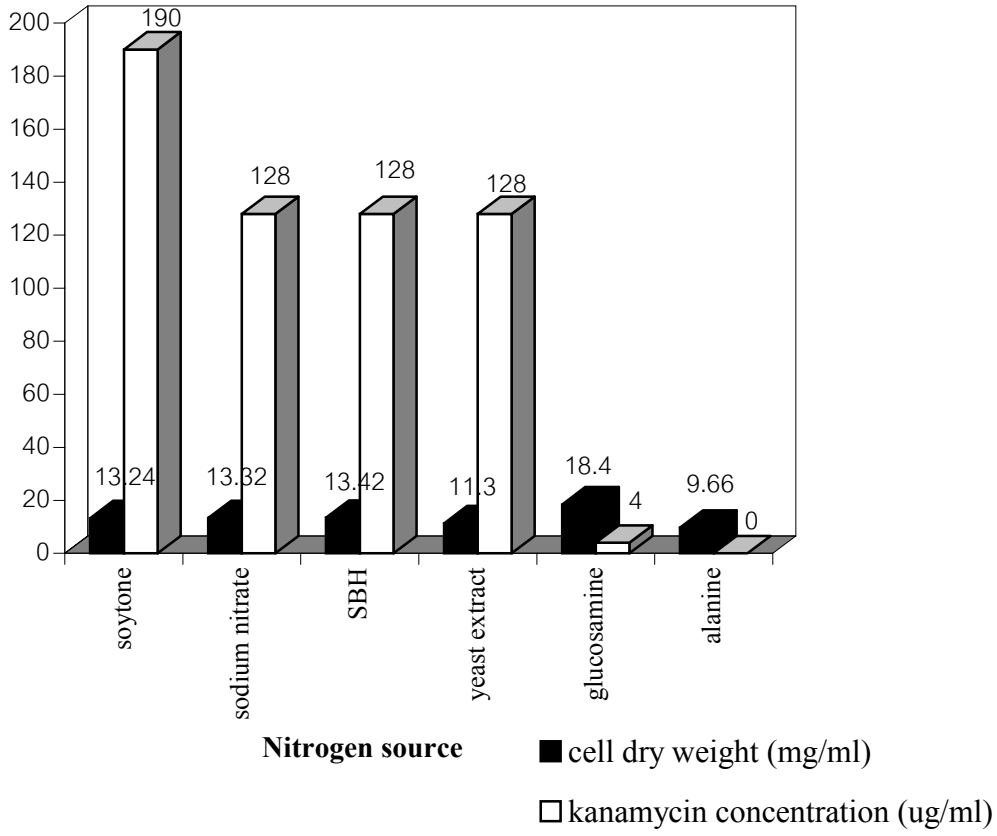


Figure 5. Effect of major N-source on kanamycin concentration and cell dry weight.

HPLC Analysis

The chromatograms of standard kanamycin sulfate of 0 and 2,000 μg/ml and a sample of kanamycin produced by UUNNK1 taken from the 5 L fermentor were shown in Fig. 6. There was a shoulder in the peak of the chromatogram of the product produced by UUNNK1 at retention time 2.86 min which was compatible with that of standard kanamycin A. When adding a mixture kanamycin sulfate of 2,000 μg/ml and culture filtrate, the shoulder of 2 peaks was combined into only one peak. Therefore, the HPLC analysis is indicated that the tested sample was kanamycin or a derivative of kanamycin.

In conclusion, with the above described optimal conditions, the mutant strain UUNNK1 could produce kanamycin yields higher than that of the original strain K1 by 25-fold. We believe that our study on optimization for kanamycin production by UUNNK1 could be useful for application at a certain level on a larger scale.

(a)

(b)

(c)

(d)

Figure 6. The chromatograms of (a) 2,000 μg/ml standard kanamycin sulfate A, (b) 0 μg/ml standard kanamycin sulfate A, (c) culture filtrate of UUNNK1 and (d) mixture of 2,000 μg/ml kanamycin sulfate A and culture filtrate of UUNNK1.

ACKNOWLEDGMENT

This work was partially supported in part by a grant from the Government Budget and partial financial support from the graduate school of Chulalongkorn University.

REFERENCES

1. Umezawa, H., Maeda, K., Ueda, M., Yagishita, K., Kondo, S., and Okam, Y. (1957) Production and isolation of new antibiotic kanamycin. *J. Antibiotics*. *Ser. A* **10(5)**, 181-188.
2. Kucers, A. (1972) The Use of Antibiotics. A Comprehensive Review with Clinical Emphasis. London: William Heinemann Med. Books Ltd., London: pp. 126-137.
3. Kattiyavara, S. (1996) Strain improvement of *Streptomyces kanamyceticus* K1 by mutation to increase kanamycin production. M.Sc. Thesis, Chulalongkorn University.
4. Johdo, O., Ishikura, T., and Yoshimoto, A. (1991) Anthracycline metabolism from *Streptomyces violaceus* A262: I. Isolation of antibiotic-blocked mutant from *Streptomyces violaceus* A262. *J. Antibiotics*. **44(10)**, 1110-1120.
5. US Government. (1987) Code of Federal Regulation. Title 21, Microbiology assay methods. Federal Printing Office, Washington, DC: 269-286.
6. Umezawa, H., Hotta, K., and Okami, Y. (1977) Elimination of the ability of a kanamycin-production strain to biosynthesize deoxystreptamine moiety by acriflavine. *J. Antibiotics* **30(12)**, 1146-1149.
7. Umezawa, H., Maeda, K., and Ueda, M. (1960) Kanamycin and process for the preparation thereof. *US Patent 2,931,798*: 935-948.
8. Reeves, D.S., and White, L.O. (1983) Principles of methods of assaying antibiotics. *Pharmaceutical Microbiology*: 140-160.
9. Gambardella, P., Punziano, M., Gionti, M., Guadalupi, C., and Mancini, G. 1985. Quantitative determination and separation of analogues of aminoglycoside antibiotics by high-performance liquid chromatography. *J.Chromatography*. **348**, 229-240.
10. Bernfeld, F. (1955) Amylase α and β. In S.P. Colowich and N.O. Kcpain (eds.), *Methods in Enzymology*. New York: Academic Press, p. 149.
11. Ashwell, G. (1966) New colorimetric methods of sugar analysis. In S.P. Colowich and N.O. Kcpain (eds.), *Methods in Enzymology*. New York: Academic Press, p. 8.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem*. 193: 265-272.
13. Drew, S.W., and Demain, A.L. (1975) Production of cephalosporin C by single and double sulfur auxotrophic mutants of

Cephalosporium acremonium. Antimicrob. Agents Chemother. **8**, 5-10.

14. Basak, K., and Majumdar, S.K. (1973) Utilization of carbon and nitrogen sources by *Streptomyces kanamyceticus* for kanamycin production. *Antimicrob. Agents Chemother*. **4**, 6-10.

Received: January 19, 1999
Accepted: September 24, 1999