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Utilization of Carbon and Nitrogen Sources by *Streptomyces kanamyceticus* for Kanamycin Production

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To select a suitable synthetic medium for kanamycin production, we tested a number of carbon and nitrogen compounds for their effect on growth of *Streptomyces kanamyceticus* ATCC 12853 as well on kanamycin production. Galactose was found to be the most suitable carbon source, though dextrin, soluble starch, and potato starch gave moderate yields. Sodium nitrate and glycine were adequate nitrogen sources for kanamycin production. There was, however, no direct relation between the growth of the organism and antibiotic formation. The pH of the medium might be an important factor for kanamycin formation, as media giving high kanamycin yields showed an alkaline pH without exception.

The study of the formation of antibiotics usually involves a search for optimal media for production. The approach is made by a systematic study of the suitability of large number of carbon sources and nitrogen sources for antibiotic formation. Early reports showed that *Streptomyces* species could utilize sugars, sugar alcohols, and some organic acids. On the basis of the utilization of different carbon sources, Pridham and Gottlieb (8) tried to characterize actinomycetes. Umezawa et al. (11) studied the carbon nutrition of *S. kanamyceticus* for kanamycin production in complex media and reported that glucose, maltose, dextrin, starch, lactose, and sucrose were better carbon sources than glycerol. Romano and Nickerson (9) studied the utilization of amino acids as carbon sources by *Streptomyces* species. Dulaney (1, 2) studied the effect of carbon and nitrogen sources for growth of *S. griseus* and streptomycin production. Majumdar and Majumdar (6) investigated the utilization of carbon and nitrogen compounds for neomycin production by *S. fradiae*. The present paper describes the utilization of carbon and nitrogen sources for growth of *S. kanamyceticus* and kanamycin production.

MATERIALS AND METHODS

The culture of *S. kanamyceticus* was maintained on maltose-sodium nitrate-mineral agar slants (5, 6) at 28 C and was subcultured at monthly intervals. The effects of different carbon and nitrogen sources were studied in the basal medium (5) consisting of: K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.04 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $ZnSO_4 \cdot 7H_2O$, 0.0005 g; and water, 1,000 ml; the pH was 7.5 ± 0.1. The carbon sources and phosphate were sterilized separately and added just prior to inoculation. For studying the effect of carbon sources on antibiotic production, sodium nitrate in 0.51% concentration was included in the basal medium; the medium was adjusted to pH 7.2 and sterilized.

For the development of inoculum, 10 ml of Lepage broth was placed in a 50-ml Erlenmeyer flask, the pH was adjusted to 7.0 ± 0.1, and the medium was sterilized. It was inoculated with a well-sporulated slant culture of *S. kanamyceticus* (10 days old) and kept on a rotary shaker (250 rpm; eccentricity, 1.27 cm) for 48 h at 28 C. A 0.5-ml portion of this broth was used to inoculate 30 ml of the fermentation medium contained in a 100-ml Erlenmeyer flask. Usually triplicate flasks were used for each test. The flasks were kept on a rotary shaker (250 rpm; eccentricity, 1.27 cm). Occasional checking of the flasks to drop adhering cells into the medium was necessary during the first 48 h of shaking. Incubation temperature was 28 C.

Determination of kanamycin potency. The filtered broth was diluted with potassium phosphate buffer (pH 8) according to Grove and Randall (3), and the estimation of kanamycin was made by a modified cup-plate method, with *Bacillus subtilis* (strain B_3) as the test organism. Kanamycin sulfate (Kantrex; Bristol Laboratories, Syracuse, N.Y.) was used as the standard, and the results are expressed in terms of micrograms of antibiotic per milliliter.

Determination of growth. Growth was determined as dry weight of cells. The mycelium was separated from the culture broth by means of suction filtration on a sintered-glass funnel (G-1) through a Whatman no. 1 filter-paper disk (3.0 cm) which had previously been dried for 24 h at 70 ± 5 C. After filtration, the paper disks were dried again at 70 ± 5 C and weighed (12). To check the variation of weight of filter paper, a blank was also prepared in each set.

A glass-electrode pH meter was used for pH measurement.

RESULTS AND DISCUSSION

Effect of carbohydrates. A number of carbohydrates were investigated for their effect on growth of *S. kanamyceticus* and on kanamycin production. D-Galactose proved to be an excellent carbon source for kanamycin formation, although soluble starch, potato starch, and maltose allowed greater amounts of growth of the organism (Table 1). It is possible that these carbon sources are utilized rapidly for the synthesis of cellular material so that little would be available as carbon and energy source for kanamycin synthesis. Galactose may be utilized less rapidly, and thus it is available during the phase of kanamycin production. Glucose, mannose, and arabinose supported abundant growth of the organism, but these sugars were poor carbon sources for kanamycin production. In case of glucose, there was a lowering of pH of the broth during the phase of antibiotic synthesis. Rapid utilization of glucose and a low pH might result in poor yields of kanamycin. It is, however, interesting to note that antibiotic formation is not solely dependent on cellular growth.

Effect of alcohol, sugar alcohols, and organic acids. Results in Table 2 show that, except for glycerol, alcohols, sugar alcohols, and organic acids were very poor carbon sources for growth of the organism as well for antibiotic formation. Glycerol supported abundant growth but resulted in a poor yield of the antibiotic.

Effect of a combination of galactose with some carbohydrates. As shown in Table 3, dextrin, starch, maltose, and glucose in combination with galactose did not result in higher antibiotic production than that obtained when galactose was used alone. However, the use of a combination of glucose and galactose raised the pH towards alkalinity, producing a kanamycin yield greater than that obtained from glucose only.

Effect of pH. To determine the effect of pH on kanamycin production, we adjusted the pH of the broth to values of 8.8 to 10.0 during the earlier phase of antibiotic synthesis. That the pH value of the broth plays an important role in kanamycin synthesis is evident from the data presented in Table 4. The acidity of the medium containing glucose as a carbon source is mainly responsible for the poor yield of kanamycin; if the pH value of the broth was adjusted to the alkaline side (pH 10) at 48 h, there was much higher antibiotic production. The adjustment of the pH of the galactose-containing medium did not, however, result in any difference in kana-

TABLE 1. *Effect of carbohydrates as carbon sources on the growth of S. kanamyceticus and kanamycin formation in a synthetic medium*[a]

Carbohydrate (1%)	3rd day			5th day			7th day		
	Growth	pH	Kanamycin	Growth	pH	Kanamycin	Growth	pH	Kanamycin
Control (no carbohydrate)	6.5	7.3	0.25	6.5	7.5	0.25	6.5	7.7	0.25
Dextrin	255	8.2	20	280	8.5	35.5	205	8.6	52
Starch (soluble)	314	8.5	6.2	307.5	9.0	17	280	9.1	25
Potato starch	410	8.3	25.2	350	8.5	28	333	8.5	34
Raffinose	40	8.0	0.8	130.5	8.2	1.2	1.35	8.5	1.0
Maltose	314	8.0	3.2	338	8.0	10	346	8.6	11
Sucrose	23.5	7.7	0	39.5	7.8	0	55	8.0	0.25
Lactose	12.5	8.0	0.35	13.5	7.7	0.37	10.5	7.7	0.32
D-Galactose	298	8.2	50	284	8.5	80	279	8.7	100
D-Glucose	271.5	8.0	9.5	126	6.8	3.75	91.5	6.2	2.0
D-Fructose	32.5	7.7	0.5	31.5	8.0	0.7	36	8.0	0.8
D-Mannose	119	8.0	5.0	263	9.1	9.5	308	9.1	15
L-(+)-Arabinose	57.5	8.0	0.7	110.5	8.2	1.0	238	8.5	1.4
D-(+)-Xylose	20.5	8.0	0	42	8.0	0	95.5	8.0	0.4

[a] Medium (30 ml per 100-ml Erlenmeyer flask) contained basal mineral salts and 0.51% sodium nitrate and was incubated on a rotary shaker at 250 rpm at 28 C. Growth is expressed as milligrams per 100 ml and kanamycin production as micrograms per milliliter.

TABLE 2. *Effect of alcohols, sugar alcohols, and sodium salts of organic acids as carbon sources on the growth of S. kanamyceticus and kanamycin formation in a synthetic medium*[a]

Carbon source (1%)	3rd day			5th day			7th day		
	Growth	pH	Kanamycin	Growth	pH	Kanamycin	Growth	pH	Kanamycin
Butanol	0	7.5	0	0	7.5	0	0	7.5	0
Glycerol	141.5	8.0	5.0	235	8.5	8.1	210	9.1	6.25
Dulcitol	10.5	7.5	0.2	11.5	7.5	0.2	3.5	8.0	0.2
Sorbitol	20.8	8.0	0.2	25.8	8.0	0.2	20	7.7	0.32
Inositol	14.5	8.0	0	15	8.0	0	5.5	8.0	0.32
Sodium succinate	14	8.0	0.8	14.5	8.0	0.63	9.5	8.2	0.28
Sodium acetate	20.5	8.2	0.5	24.5	8.2	0.1	14.5	8.2	0.63
Sodium citrate	11.5	8.0	0.25	10.0	8.0	0.35	5.5	8.0	0.71

[a] Cultivation conditions as in Table 1. Growth is expressed as milligrams per 100 ml and kanamycin production as micrograms per milliliter.

TABLE 3. *Effect of a combination of galactose with some carbohydrates on kanamycin production by S. kanamyceticus in a synthetic medium*[a]

Carbohydrates	3rd day		5th day		7th day	
	pH	Kanamycin	pH	Kanamycin	pH	Kanamycin
Galactose (0.5%) + 0.5% dextrin	8.2	42	8.5	71.6	9.1	75
Galactose (0.5%) + 0.5% starch (soluble)	8.0	36	8.5	36.4	9.0	40
Galactose (0.5%) + 0.5% maltose	8.2	27.1	8.5	41.4	9.1	55
Galactose (0.5%) + 0.5% glucose	8.2	47.2	8.5	49	9.1	68
Galactose (1%)	8.2	50	8.5	80	8.7	100

[a] Cultivation conditions as in Table 1. Kanamycin production is expressed as micrograms per milliliter.

TABLE 4. *Effect of pH on kanamycin production by S. kanamyceticus in a synthetic medium containing glucose or galactose as a single carbon source*[a]

Carbon sources (1%)	48 h		72 h			120 h		168 h	
	pH	Adjusted pH	pH	Adjusted pH	Kanamycin	pH	Kanamycin	pH	Kanamycin
Control (pH not adjusted)	8.0	—	8.2	—	9.5	6.8	3.5	6.2	2.0
Glucose	8.0	9.0	8.5	—	9.0	8.4	28	8.5	30.2
	8.0	9.0	8.5	9.2	9.0	8.4	28	8.5	30.0
	8.0	10	8.8	—	11	8.5	31.6	8.5	39.6
	8.0	10	8.8	9.2	11	8.8	14	8.4	15.8
Control (pH not adjusted)	8.0	—	8.2	—	50	8.5	80	8.7	100
Galactose	8.0	—	8.2	8.8	55	8.4	75	8.7	105
	8.0	—	8.2	9.1	60	8.5	80	8.7	108

[a] Cultivation conditions as in Table 1. Kanamycin production is expressed as micrograms per milliliter.

mycin yield. It is possible that pH changes of the broth during galactose utilization are favorable for kanamycin production. This also confirms the observation made by Umezawa et al. (11) that the pH range of 8.0 to 8.6 is optimal for kanamycin synthesis. It was reported earlier by Sebek (10) that little or almost no neomycin synthesis took place at a pH below 7.0. Majumdar and Majumdar (7) also reported that alkaline pH favored neomycin production. The influence of pH on kanamycin production may be explained in a similar manner.

As galactose is an excellent carbon source for kanamycin production by *S. kanamyceticus*, different levels of galactose were tested to determine the optimal concentration for kanamycin

TABLE 5. *Effect of different amino acids and inorganic nitrogen sources on the growth of S. kanamyceticus and kanamycin formation in a synthetic medium*[a]

Nitrogen source	5th day			7th day		
	pH	Growth	Kanamycin	pH	Growth	Kanamycin
L-Aspartic acid	8.5	380	10	8.6	570	30
L-Glutamic acid	8.4	420	2.5	8.6	530	10
L-Asparagine	8.2	350	2.5	8.3	394	7.5
DL-Serine	6.5	56.5	0	7.2	154	0.5
Glycine	8.4	263	40	8.5	540	80
DL-Alanine	8.4	440	7.9	8.2	470	3.15
DL-Threonine	7.7	44	5	8.0	85.5	9.5
L-Lysine	8.4	263	10	8.3	290	10
DL-Valine	7.1	142.5	3.2	7.2	209	3.35
L-Histidine	8.0	570	11.2	8.4	441	13.5
DL-Methionine	7.0	50	0	7.1	70	0
L-Proline	7.2	330	2.5	7.4	458	1.26
DL-Phenylalanine	6.4	120	0	6.4	140	0
DL-Tryptophan	6.7	52	0	6.9	27	0
L-Cystine	6.7	75	0	6.5	54	0
L-Leucine	6.9	150	0.5	7.2	210	1.5
L-Arginine	8.6	365	11.2	8.8	455	12.6
Sodium nitrate	8.5	583	100	8.7	453	150
Ammonium chloride	6.7	50	0	6	40	0
Ammonium sulfate	6.2	50	0	6.5	44.5	0
Ammonium nitrate	6.0	30	0	6.4	25	0
Urea	8.4	50	2	8.1	30	0
Ammonium hydrogen phosphate (K_2HPO_4 omitted)	6.7	101	4	6.4	120	7.9
Ammonium hydrogen phosphate (plus K_2HPO_4)	6.7	230	1.25	6.4	210	0

[a] Cultivation conditions as in Table 1. Nitrogen sources were added at 84 mg of N per 100 ml. Growth is expressed as milligrams per 100 ml and kanamycin production as micrograms per milliliter.

production. Galactose at a concentration of 2 g/100 ml gave maximal antibiotic titers; a lower or higher dose decreased the yield.

Effect of different nitrogen sources. The medium for testing different nitrogen sources contained the basal mineral salts plus 2% galactose. The amino acids and inorganic nitrogen compounds were employed at a concentration equivalent to 84 mg of nitrogen per 100 ml. Highest kanamycin yield was obtained in a synthetic medium containing sodium nitrate as the nitrogen source (Table 5). It was also observed that amino acids with the exception of glycine were poor nitrogen sources for kanamycin production, although some of the amino acids supported abundant growth of the organism. Methionine, phenylalanine, tryptophan, cystine, and some inorganic salts yielded no antibiotic, and poor growth was observed in most cases. The acidity of the medium may be responsible for poor growth of the organism and antibiotic production.

The optimal nitrogen concentration for kanamycin production was found to be 84 mg per 100 ml.

The suitable synthetic medium selected as a result of the present study consisted of: galactose, 20 g/liter; sodium nitrate, 5.1 g/liter; and inorganic salts (Materials and Methods). This medium yielded 150 μg of kanamycin per ml.

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